

TRANSPORT VECTORSRelated Applications

- 5 This application claims priority to the following Great Britain applications:
GB9825000.4, GB9825001.2 (filed on 13 November 1998), GB 9902525.6, GB 9902522.3 (filed on 4 February 1999, and GB 9914578.1 (filed on 22 June 1999), the contents of which are hereby incorporated by reference.

Background

- 10 The present invention relates to novel membrane translocation peptide carrier moieties and membrane translocation vectors comprising a novel peptide carrier moiety together with a cargo moiety, of use in the improved delivery of therapeutic agents into target cells.
- 15 The pharmaceutical industry has for many years concerned itself with the efficient delivery of therapeutic agents. This problem may be attributed to the short clearance time of the agent in the body (short half-life), the location of the site of action or possibly the nature of the therapeutic agent itself, for example, its solubility, hydrophobicity etc.. Thus, many developments and strategies have been adopted,
- 20 including formulating the therapeutic agent so as to protect it from a hostile environment on route to its site of action; by for example, enterically coated tablets, controlled release devices and the like.
- 25 The development of peptide derived therapeutic agents has posed a further problem due their susceptibility to enzymatic degradation not only in the GI tract but also in the bloodstream. An example of how this problem has been addressed relates to the incorporation of the peptides into liposomes or polymeric microspheres that target the peptides to the lymph system.

A further related problem, especially for therapeutic agents that function intracellularly is the barrier posed by the cell membrane. Thus, it may be possible to increase the half life of the agent or ensure that it passes through the body without being degraded, but many agents must actually enter cells to exert their therapeutic effect.

Homeoproteins are trans-activating factors involved in multiple morphological processes. They bind to DNA through a sequence of 60 amino acid residues, the so-called homeodomain. The structure of this domain consists of three α -helices, interrupted by a β -turn between helices 2 and 3 (Gehring, W.J. et al., (1990) Trends Genet 6, 323-9). The phylogenetic relationship between numerous homeoproteins is striking at the level of the homeodomain and particularly within the third α -helix. This helix is responsible for both the interaction with DNA, as well as the capacity of homeoproteins to translocate across cell membranes to cell nuclei in a non-specific manner.

European Patent 485578 discloses that the homeodomain and specifically, helix 3 of a homeobox peptide, particularly that derived from the Drosophila Antennapedia, is of use as an intracellular transport vector. The patent disclosed that a specific 57 amino acid sequence of a Drosophila Antennapedia homeopeptide (referred to as the pAntp peptide) was capable of penetrating fibroblasts and embryo cells (in vivo). Emphasis was placed upon the last 27 amino acids of the sequence that correspond with the helix 3 and 4. There is no description of the pAntp peptide being linked to any other peptide or therapeutic agent.

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Subsequent disclosures (Derossi D et al., J Biol Chem (1994) 269, 10444-10450, Derossi D et al., J Biol Chem (1996) 271, 18188-18193, Joliot AH et al., (1991) The New Biol 3, 1121-1134 and PNAS (1991) 88, 1864-1868, Perez F et al., J Cell Sci (1992) 102, 712-722), have focused on a 16 amino acid synthetic peptide derived from the third helix of the Antennapedia homeodomain that may be used for

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the intracellular delivery of bioactive products and antisense oligonucleotides. The amino acid sequence of this peptide is RQIKIWFQNRRMKWKK (SEQ ID No. 1) also known as penetratin. In the course of their investigations the above authors synthesized several variants on this sequence, these corresponding to residues 41-60, 5 41-55 and 46-60 of the pAntp peptide and showed that in all cases, the only peptides to internalise into the cells were those that included the residues 43-58 (Derossi D et al., supra.).

In an effort to prevent the enzymatic cleavage of this peptide Brugidou J et al., 10 (Biochem Biophys Res Comm (1995) 214(2), 685-693) prepared a *retro-inverso* form (D amino acids in reverse order) of SEQ ID No. 1, substituting the two isoleucine residues at positions 3 and 5 of penetratin with valine and adding a glycine residue at the C-terminus to facilitate binding to a resin. A further *retro-inverso* form was prepared replacing the extra glycine with a cholesterol moiety attached via a sulfhydryl 15 linker group. The addition of the cholesterol moiety improved penetration due to the increased hydrophobicity of the molecule.

This development of the *retro-inverso* form of penetratin has given rise to WO 97/12912 that discloses peptides of 16 amino acids comprising between 6 and 10 20 hydrophobic amino acids wherein the sixth amino acid from either end must be tryptophan. This disclosure attempts to define the minimal characteristics of sequences capable of acting as internalisation vectors as being the retention of a tryptophan residue at the sixth position from the amino terminus and that the peptide contains from 6 to 10 hydrophobic amino acid residues (the classification of 25 hydrophobic amino residues in WO97/12912 is not believed to be in agreement with the generally accepted classification).

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From the disclosures discussed above, as summarised in WO97/12912, it has been concluded that essential to the membrane translocating properties of the 30 homeodomain peptides, is the presence of a tryptophan residue as the sixth residue

from the amino terminus. Conforming to these requirements has been a penetratin variant of the formula (KWKK)₄ which has been described as having translocating ability (Maruta H et al. Cytoskeletal tumour suppressors that block oncogenic RAS signalling. Presented at Anti-Cancer Proteins and Drugs: Structure, Function and Design; 6 - 9 November 1998, New York Academy of Sciences. Poster/abstract No. 11) and Plank C et. al. (Human Gene Therapy, (1998) 10, 319-332) that discloses a number of branched membrane translocating peptides such as (KWKK)₂KGGC, wherein each KWKK is joined to the following lysine residue.

10 Summary

The present invention seeks to provide a wider range of membrane translocating peptides based on penetratin, including peptides that do not contain a tryptophan residue as the sixth residue from the amino terminus and peptides that are smaller in size than penetratin. Such smaller or truncated forms of penetratin are advantageous in that they are more acceptable to the pharmaceutical industry as delivery carrier moieties, by virtue of the carrier-cargo conjugate having a advantageous immunogenicity, solubility and clearance and in some cases advantageous efficacy as compared to using a conjugate comprised of "full length" penetratin (SEQ ID No. 1). Thus, a first aspect of the present invention relates to truncated penetratin derivatives, whereas a second aspect relates to modified forms of penetratin. These first and second aspects are described below in greater detail and are both hereinafter referred to as the "carrier moiety" of a cargo delivery system.

A first aspect of the present invention therefore relates to a membrane translocation peptide carrier moiety of formula;

RQIKIWFQNRRMKWKK (SEQ ID No. 1)

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Within the above definitions of the peptide carrier moieties of the present invention, the specific amino acid residues of the peptide may be modified in such a manner that retains their ability to translocate, such modified peptides are referred to as

5 "variants".

A variant of a carrier moiety as defined above includes any variation wherein;

(a) one or more amino acid residues are replaced by a naturally or non-naturally occurring amino acid residue (b) the order of two or more amino acid residues is

10 reversed, (c) both (a) and (b) are present together, (d) a spacer group is present between any two amino acid residues, (e) one or more amino acid residues are in peptoid form, (f) the (N-C-C) backbone of one or more amino acid residues of the peptide has been modified, or any of (a)-(f) in combination. Preferably, the variants arise from one of (a), (b) or (c).

15 Thus, homologous substitution (substitution and replacement are both used herein to mean the interchange of an existing amino acid residue, with an alternative residue) may occur i.e. like-for-like substitution such as basic for basic, acidic for acidic, polar for polar etc. Non-homologous substitution may also occur i.e. from one

20 class of residue to another or alternatively involving the inclusion of unnatural amino acids such as ornithine (hereinafter referred to as Z), diaminobutyric acid ornithine (hereinafter referred to as B), norleucine ornithine (hereinafter referred to as O), pyriylalanine, thienylalanine, naphthylalanine and phenylglycine, a more detailed list of which appears below. Within each peptide carrier moiety more than one amino acid

25 residue may be modified at a time.

As used herein, amino acids are classified according to the following classes;

basic; H. K, R

30 acidic; D. E

non-polar; A, F, G, I, L, M, P, V, W

polar; C, N, Q, S, T, Y,

(using the internationally accepted single letter amino acid notation)

5 and homologous and non-homologous substitution is defined using these classes. Thus, homologous substitution is used to refer to substitution from within the same class, whereas non-homologous substitution refers to substitution from a different class or by an unnatural amino acid.

10 Suitable spacer groups that may be inserted between any two amino acid residues of the carrier moiety include alkyl groups such as methyl, ethyl or propyl groups in addition to amino acid spacers such as glycine or β -alanine residues. A further form of variation, type (e), involving the presence of one or more amino acid residues in peptoid form, will be well understood by those skilled in the art. For the
15 avoidance of doubt, "the peptoid form" is used to refer to variant amino acid residues wherein the α -carbon substituent group is on the residue's nitrogen atom rather than the α -carbon. Processes for preparing peptides in the peptoid form are known in the art, for example Simon RJ et al., PNAS (1992) 89(20), 9367-9371 and Horwell DC, Trends Biotechnol. (1995) 13(4), 132-134. Type (f) modification may occur by
20 methods such as those described in International Application PCT/GB99/01855.

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25 Within the definition of formula (I) it has been demonstrated that it is preferable for amino acid variation, preferably of type (a) or (b), to occur independently at any of positions 1, 2, 3, 5 or 6. More preferably, amino acid variation occurs at positions 3 or 7, especially 3. Homologous substitution has been found to be preferable at positions 1 and 2, whereas positions 3, 4, 5 and 6 have surprisingly been observed to accept non-homologous substitution. As mentioned above more than one homologous or non-homologous substitution may occur simultaneously, for example at positions 2 and 3, 4 and 5 or 5 and 6. Further variation may occur by virtue of
30 reversing the sequence of a number of amino acid residues within a sequence. For

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example in the peptide sequence RRMKWKK, the lysine and tryptophan residues may be reversed to give a peptide RRMWKKK. This modification may additionally occur in combination with a homologous or non-homologous substitution, for example, the sequence RROKWKK giving rise to RROWKKK.

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The carrier moiety may include further amino acid residues at the amino terminal end, more preferably by the addition of from 1 to 3 amino acid residues. Thus, a further embodiment of this aspect of the present invention relates to a peptide selected from RRMKWKK, NRRMKWKK, QNRRMKWKK and FQNRRMKWKK.

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In the most preferred embodiment of the first aspect of the invention, the truncated form of penetratin is of formula (I) described above or more preferably to a 7 amino acid peptide selected from KRMKWKK, RKMKWKK, RREKWKK, RRQKWKK, RROKWKK, RRMKQKK, RRMKWFK, RORKWKK, RRMWKKK, RROWKKK, RRMKKWK and RROKKWK, most preferably, the peptide carrier moiety is RRMKWKK.

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A second aspect of the present invention relates to a membrane translocation peptide carrier moiety of formula;

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RQIKIWFQNRRMKWKK (SEQ ID No. 1)

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wherein at least one amino acid residue is replaced by an alternative natural or unnatural replacement amino acid residue.

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In a preferred embodiment of the second aspect of the present invention, the sixth amino acid residue from the amino terminus of the peptide is not tryptophan. As will be described below, it has been demonstrated that the prevalent accepted principle

that tryptophan must be present at this position is unfounded and hence a wider range of membrane translocating peptides has been identified.

In a preferred embodiment, the peptide carrier moieties of the present invention includes compounds 21 to 36 in (SEQ ID Nos.) shown in Table 3 below where they are shown together with a biotinyl- β Ala handle used for the purposes of biochemical assay. In a more preferred embodiment the peptide is compound such as compound 26, wherein the sixth amino acid from the amino terminus is not tryptophan.

In one embodiment the replacement amino acid residue is selected from the residues of alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. The replacement amino acid residue may additionally be selected from unnatural amino acids. Within the above definitions of the peptide carrier moieties of the present invention, the specific amino acid residues of the peptide may be modified in such a manner that retains their ability to translocate, such modified peptides are referred to as "variants".

A variant of a carrier moiety as defined above includes any variation wherein;

(a) one or more amino acid residues are replaced by a naturally or non-naturally occurring amino acid residue (b) the order of two or more amino acid residues is reversed, (c) both (a) and (b) are present together, (d) a spacer group is present between any two amino acid (e) one or more amino acid residues are in peptoid form, (f) the (N-C-C) backbone of one or more amino acid residues of the peptide has been modified, or any of (a)-(f) in combination. Preferably, the variants arise from one of (a), (b) or (c).

Thus, homologous substitution (substitution and replacement are both used herein to mean the interchange of an existing amino acid residue, with an alternative residue) may occur i.e. like-for-like substitution such as basic for basic, acidic for

acidic, polar for polar etc. Non-homologous substitution may also occur i.e. from one class of residue to another or alternatively involving the inclusion of unnatural amino acids such as ornithine (hereinafter referred to as Z), diaminobutyric acid ornithine (hereinafter referred to as B), norleucine ornithine (hereinafter referred to as O), pyriylalanine, thienylalanine, naphthylalanine and phenylglycine. Within each peptide carrier moiety more than one amino acid residue may be modified at a time.

As used herein, amino acids are classified according to the following classes;

10 basic; H, K, R

acidic; D, E

non-polar; A, F, G, I, L, M, P, V, W

polar; C, N, Q, S, T, Y,

(using the internationally accepted single letter amino acid notation)

15 and homologous and non-homologous substitution is defined using these classes. Thus, homologous substitution is used to refer to substitution from within the same class, whereas non-homologous substitution refers to substitution from a different class or by an unnatural amino acid.

20 Suitable spacer groups that may be inserted between any two amino acid residues of the carrier moiety include alkyl groups such as methyl, ethyl or propyl groups in addition to amino acid spacers such as glycine or β -alanine residues. A further form of variation, type (e), involving the presence of one or more amino acid residues in peptoid form, will be well understood by those skilled in the art. For the avoidance of doubt, "the peptoid form" is used to refer to variant amino acid residues wherein the α -carbon substituent group is on the residue's nitrogen atom rather than the α -carbon. Processes for preparing peptides in the peptoid form are known in the art. for example Simon RJ et al., PNAS (1992) 89(20), 9367-9371 and Horwell DC, Trends Biotechnol. (1995) 13(4), 132-134.

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Further non-natural amino acid derivatives that may be used in the context of the either the first or second aspects of the present include; α^* and α -disubstituted* amino acids, N-alkyl amino acids*, lactic acid*, halide derivatives of natural amino acids such as trifluorotyrosine*, p-Cl-phenylalanine*, p-Br-phenylalanine*, p-I-phenylalanine*, L-allyl-glycine*, β -alanine*, L- α -amino butyric acid*, L- γ -amino butyric acid*, L- α -amino isobutyric acid*, L- ϵ -amino caproic acid[#], 7-amino heptanoic acid*, L-methionine sulfone[#], L-norleucine*, L-norvaline*, p-nitro-L-phenylalanine*, L-hydroxyproline[#], L-thioprolin*, methyl derivatives of phenylalanine (Phe) such as 4-methyl-Phe*, pentamethyl-Phe*, L-Phe (4-amino)[#], L-Tyr (methyl)*, L-Phe (4-isopropyl)*, L-Tic (1,2,3,4-tetrahydroisoquinoline-3-carboxyl acid)*, L-diaminopropionic acid[#] and L-Phe (4-benzyl)*. The notation * has been utilised for the purpose of the discussion above, to indicate the hydrophobic nature of the derivative whereas # has been utilised to indicate the hydrophilic nature of the derivative, #* indicates amphipathic characteristics.

The peptide carrier moieties of the present invention may comprise amino acids in the L or D form, i.e. one or more residues, preferably all the residues may be in the L or D form. Within this embodiment, the peptide may be in the *retro* form for example, the peptide KKWKORR.

In a preferred embodiment of the present invention, the sixth amino acid residue from the amino terminus of the peptide is not tryptophan.

The membrane translocation peptides of the present invention are capable of translocating the cell membrane and in a preferred embodiment, also the nuclear membrane. It is irrelevant whether the peptide translocates from the exterior of the cell/nucleus or from the interior, i.e. the peptides may originate within the cytoplasm or nucleus (for example, by virtue of having being synthesised there or inserted into that compartment), and translocate to a location exterior to the cellular compartment (cytoplasm or nucleus) it originates from. In general, the peptides are prepared outside

the cell and translocate from an exterior location to the cytoplasm and then optionally, on into the nucleus.

As used herein, the term "cell membrane translocation" refers to the ability of the peptide to cross the cell membrane and enter the cytosol/cytoplasm of a cell or to cross from the cytosol/cytoplasm of a cell to the exterior, extra-cellular or interstitial space.

The term "nuclear membrane translocation" refers to the ability of the peptide to cross the membrane structure surrounding the cell nucleus, or to cross from the cytosol/cytoplasm of a cell to the nucleus.

Brief Description of the Figures

Figure 1 shows the RP-HPLC analysis of some of the peptide carrier moieties prepared in accordance with the first aspect of the present invention.

Figure 2 shows the results of the cell internalisation assay performed using peptide carrier moieties prepared in accordance with the first aspect of the invention.

Figure 3 shows the results of the cell internalisation assay performed using compound 39 (fluorescein labelled Penetratin).

Figure 4 (A, B and C) show real-time visualisation of the cell internalisation assay performed using compound 39.

Figure 5 shows the results of the cell internalisation assay performed using peptide carrier moieties prepared in accordance with the second aspect of the present invention.

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Detailed Description

In a further aspect of the invention, a peptide carrier moiety (either the truncated or modified form of penetratin in accordance with either the first or second aspects) is linked to a cargo moiety to form a cell translocation vector. The cargo moiety may comprise oligonucleotides, nucleotides, proteins, peptides, biologically active compounds, diagnostic agents or combinations thereof.

In a preferred embodiment the cargo moiety is a protein or peptide and in a more preferred embodiment the cargo moiety is a biologically active agent such as a drug.

The cargo moiety may be directly or indirectly linked to the carrier moiety. In the embodiment wherein the cargo moiety is indirectly linked to the carrier, the linkage may be by an intermediary bonding group such as a sulphhydryl or carboxyl group or any larger group, all such linking groups are herein referred to as linker moieties as discussed below. Preferably, the carrier and cargo moieties are linked directly.

Examples of suitable oligonucleotide cargo moieties include genes, gene fragments, sequences of DNA, cDNA, RNA, nucleotides, nucleosides, heterocyclic bases, synthetic and non-synthetic, sense or anti-sense oligonucleotides including those with nuclease resistant backbones etc. or any of the above incorporating a radioactive label, that are desired to be delivered into a cell or alternatively to be delivered from a cell to its exterior. Preferably, the oligonucleotide cargo moiety is a gene or gene fragment.

Examples of suitable protein or peptide cargo moieties include; proteins, peptides, and their derivatives such as: antibodies and fragments thereof; cytokines and derivatives or fragments thereof, for example, the interleukins (IL) and especially the IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11 and IL-12 subtypes thereof; colony stimulating factors, for example granulocyte-macrophage colony stimulating factor, granulocyte-colony stimulating factor (alpha and beta forms), macrophage colony stimulating factor (also known as CSF-1); haemopoietins, for example erythropoietin, haemopoietin-alpha and kit-ligand (also known as stem cell factor or Steel factor); interferons (IFNs), for example IFN- α , IFN- β and IFN- γ ; growth factors and bifunctional growth modulators, for example epidermal growth factor, platelet derived growth factor, transforming growth factor (alpha and beta forms), amphiregulin, somatomedin-C, bone growth factor, fibroblast growth factors, insulin-like growth factors, heparin binding growth factors and tumour growth factors; differentiation factors and the like, for example macrophage differentiating factor, differentiation inducing factor (DIF) and leukaemia inhibitory factor; activating factors, for example platelet activating factor and macrophage activation factor; coagulation factors such as fibrinolytic/anticoagulant agents including heparin and proteases and their pro-factors, for example clotting factors VII, VIII, IX, X, XI and XII, antithrombin III, protein C, protein S, streptokinase, urokinase, prourokinase, tissue plasminogen activator, fibrinogen and hirudin; peptide hormones, for example insulin, growth hormone, gonadotrophins, follicle stimulating hormone, leutenising hormone, growth hormone releasing hormone and calcitonin; enzymes such as superoxide dismutase, glucocerebrosidase, asparaginase and adenosine deaminase; vaccines or vaccine antigens such as, for example hepatitis-B vaccine, malaria vaccine, melanoma vaccine and HIV-1 vaccine; transcription factors and transcriptional modulators. More preferably, the cargo may be a protein or peptide selected from proteins or peptides that interfere with the cell cycle, such as p53 peptides or fragments thereof, p21^{WAF} peptides or fragments thereof such as those described in WO96/14334 and WO97/42222, Fen1 peptides or fragments

thereof such as those described in WO96/35715, p16 peptides or fragments thereof such as those described in WO97/11174 and fragments and derivatives thereof

Examples of a suitable non-nucleotide/proteinaceous biologically active cargo moieties are drug moieties selected from cytotoxic agents, anti-neoplastic agents, anti-hypertensives, cardioprotective agents, anti-arrhythmics, ACE inhibitors, anti-inflammatory's, diuretics, muscle relaxants, local anaesthetics, hormones, cholesterol lowering drugs, anti-coagulants, anti-depressants, tranquilizers, neuroleptics, analgesics such as a narcotic or anti-pyretic analgesics, anti-virals, anti-bacterials, anti-fungals, bacteriostats, CNS active agents, anti-convulsants, anxiolytics, antacids, narcotics, antibiotics, respiratory agents, anti-histamines, immunosuppressants, immunoactivating agents, nutritional additives, anti-tussives, diagnostic agents, emetics and anti-emetics, carbohydrates, glycosoaminoglycans, glycoproteins and polysaccharides; lipids, for example phosphatidyl-ethanolamine, phosphatidylserine and derivatives thereof; sphingosine; steroids; vitamins; antibiotics including lantibiotics; bacteristatic and bactericidal agents; antifungal, anthelmintic and other agents effective against infective agents including unicellular pathogens; small effector molecules such as noradrenalin, alpha adrenergic receptor ligands, dopamine receptor ligands, histamine receptor ligands, GABA/benzodiazepine receptor ligands, serotonin receptor ligands, leukotrienes and triodothyronine; cytotoxic agents such as doxorubicin, methotrexate and derivatives thereof.

Preferably the drug moiety is a cytotoxic or anti-neoplastic agent, particularly those which are used for cancer therapy. Such drugs include, in general, DNA damaging agents, anti-metabolites, anti-tumour antibiotics, natural products and their analogues, dihydrofolate reductase inhibitors, pyrimidine analogues, purine analogues, cyclin-dependent kinase inhibitors, thymidylate synthase inhibitors, DNA intercalators, DNA cleavers, topoisomerase inhibitors, anthracyclines, vinca drugs, mitomycins, bleomycins, cytotoxic nucleosides, pteridine drugs, diynenes, podophyllotoxins, platinum containing drugs, differentiation inducers, and taxanes. Particularly useful

members of those classes include, for example, methotrexate, methopterin, dichloromethotrexate, 5-fluorouracil, 6-mercaptopurine, tri-substituted purines such as olomoucine, roscovitine and bohemine, flavopiridol, staurosporin, cytosine arabinoside, melphalan, leurosine, actinomycin, daunorubicin, doxorubicin, mitomycin D, mitomycin A, carinomycin, aminopterin, tallysomycin, podophyllotoxin, etoposide, cisplatin, carboplatinum, vinblastine, vincristine, vindesin, paclitaxel, docetaxel, taxotere retinoic acid, butyric acid, acetyl spermidine, tamoxifen, irinotecan and camptothecin. Most preferably the drug is selected from olomoucine, roscovitine and bohemine, flavopiridol, staurosporin, and podophyllotoxin, etoposide, purvalanol derivatives, taxol, paclitaxel and camptothecin.

As discussed above the drug and carrier moieties may be linked directly or indirectly via a linker moiety. Direct linkage may occur through any convenient functional group on the drug moiety such as a hydroxy, carboxy or amino group. Indirect linkage which is preferable, will occur through a linking moiety. Suitable linking moieties include bi- and multi-functional alkyl, aryl, aralkyl or peptidic moieties, alkyl, aryl or aralkyl aldehydes acids esters and anyhydrides, sulphydryl or carboxyl groups, such as maleimido benzoic acid derivatives, maleimido propionic acid derivatives and succinimido derivatives or may be derived from cyanuric bromide or chloride, carbonyldiimidazole, succinimidyl esters or sulphonic halides and the like. The functional groups on the linker moiety used to form covalent bonds between linker and drugs on the one hand, as well as linker and carrier moiety on the other hand, may be two or more of, e.g., amino, hydrazino, hydroxyl, thiol, maleimido, carbonyl, and carboxyl groups, etc. The linker moiety may include a short sequence of from 1 to 4 amino acid residues that optionally includes a cysteine residue through which the linker moiety bonds to the carrier moiety.

In accordance with the present invention each carrier moiety may be linked to at least one drug moiety. In a further embodiment, the carrier moiety is prepared such as to facilitate linkage to more than one cargo moiety, each cargo moiety being the

same or different. For example, the carrier moiety may comprise components that themselves facilitate the attachment of more than one cargo moiety such as derivatives of naturally occurring amino acids or insertion of a multi-valent synthetic amino acid, or it may be specifically adapted to do so for example by a network of branched lysine residues that may be attached to the carrier moiety as a linking group and each lysine residue may then be attached to a cargo moiety. In this manner a single carrier moiety may carry up to 32 cargo moieties, preferably from 2 to 10 or more preferably from 4 to 5 cargo moieties. In this further embodiment each cargo moiety may be directly or indirectly linked to the carrier moiety. When more than one different type of cargo moiety is attached, it is possible to co-ordinate the ratios and dosages of the individual drugs to facilitate the administration of specific cargo combinations.

In a preferred example of this embodiment, the carrier moiety is peptide carrier moiety as defined above, with a network of lysine residues attached to at least one end facilitating the attachment of up to 32 cargo moieties.

In a further embodiment, the translocation vector may further comprise a targeting moiety. The targeting moiety is capable of directing the carrier moiety to the specific cell type to which it is preferable for the cargo moiety to function. Thus, the targeting moiety acts as an address system biasing the bodies natural distribution of drugs or the delivery system to a particular cell type. The targeting moiety may be attached to the cargo moiety or more preferably to the carrier moiety and will direct the delivery system to a desired site, upon arrival at which the carrier moiety will facilitate the cellular internalisation of the cargo. Suitable targeting moieties include the peptide sequences identified by E Ruoslahti et al. in US Patent 5,622,699; Pasqualini, R. Ruoslahti, E. Nature (London) (1996), 380, 364-366, Ruoslahti, E. Ann. Rev. Cell Dev. Biol. (1996), 12, 697-715; Arap, W, Pasqualini, R, Ruoslahti, E, Science (1998), 279, 377-380. These disclosures, which are herein incorporated by reference, describe certain peptides that have been found to act as address labels to certain cell types.

In accordance with any of the above defined embodiments of the present invention, the amino acids (any number thereof, but preferably all of them) comprising the peptide carrier moiety may be in the L or D (*inverso*) form. More preferably they are in the L form.

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In a further embodiment the carrier moiety as previously described may be in the *retro* form. Within this further embodiment, the amino acids (any number thereof, but preferably all of them) comprising the peptide carrier moiety may be in the L or D form.

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When the cargo moiety is itself a protein or peptide, the carrier and cargo moieties may both be in the L or D forms or alternatively the carrier may be in the L form and the cargo in the D form or the carrier in the D form and the cargo in the L form.

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Within the carrier moieties defined as penetratin or derivatives thereof, a further modification that is beneficial in the context of the present invention is conversion of the free carboxyl group of the carboxy terminal amino acid residue, to an carboxamide group. By way of example, when the carrier moiety is of formula I (RRMKWKK) the carboxy terminal lysine residue may have its carboxyl group converted into an carboxamide group. This modification is believed to enhance the stability of the carrier moiety and hence the delivery system as a whole. Thus, the C-terminal amino acid residue may be in the form -C(O)-NRR', wherein R and R' are each independently selected from hydrogen, C1-6 alkyl, C1-6 alkylene or C1-6 alkynyl (collectively referred to "alk"), aryl such as benzyl or alkaryl, each optionally substituted by heteroatoms such as O, S or N. Preferably at least one of R or R' is hydrogen, most preferably, they are both hydrogen.

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Thus, its most preferred embodiment, the present invention relates to a carrier moiety RRMKWKK with an optionally amidated terminal lysine residue, directly

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#	Drug moiety	Linker moiety	Carrier moiety
	paclitaxel	2'-succinimidopropionoyl-CβA	RRMKWKK-NH ₂
	podophyllotoxin	4-succinimidopropionoyl-CβA	RRMKWKK-NH ₂
	podophyllotoxin	4-succinimidopropionoyl-CβA	(D-R)(D-R)(D-M)(D-K)(D-W)(D-K)(D-K-NH ₂)
	epipodophyllotoxin	4'-succinimidopropionoyl-CβA	RRMKWKK-NH ₂
	podophyllotoxin	4-acetyl-CβA	RRMKWKK-NH ₂
	4'-demethyl epipodophyllotoxin	4-acetyl-CβA	RRMKWKK-NH ₂
	podophyllotoxin	4-succinimidopropionoyl-GCβA	RRMKWKK-NH ₂
C-term	podophyllotoxin	4-succinimidopropionoyl-C	RRMKWKK
N-term	podophyllotoxin	4-succinimidopropionoyl-C	
N-term	epipodophyllotoxin	4'-succinimidopropionoyl-C	RRMKWKK
C-term	camptothecin	10-O-succinimidopropionoyl-C	
N-term	epipodophyllotoxin	4'-succinimidopropionoyl-C	RRMKWKK
C-term	paclitaxel	2'-(succinimido)propionoyl-C	
	4'-methoxy-epipodophyllotoxin	4-(4''-aminoanilino)succinimidopropionoyl-CβA	RRMKWKK-NH ₂
	4'-demethyl-epipodophyllotoxin	4-(4''-aminoanilino)succinimidopropionoyl-CβA	RRMKWKK-NH ₂

The therapeutic effect resulting from the administration of the delivery system may arise from the intact delivery system or any of its dissociated components that include the cargo moiety i.e the cargo moiety alone or bound to the linker, part of the linker or the linker and part of the carrier. Thus the term "delivery system" has been used herein to have its ordinary meaning i.e that of delivering something such as the cargo moiety and additionally to include the system or any portion thereof as being active in its intact state. Thus, the benefits provided by the system discussed above are applicable to the cargo and delivery system.

The delivery vectors may be prepared by any methods known in the art. For example, the carrier moiety peptide can be assembled using conventional solution- or solid-phase peptide synthesis methods, affording a fully protected precursor with only the terminal amino group in deprotected reactive form. This function can then be reacted directly with a cargo moiety or a suitable reactive derivative of a cargo moiety. Alternatively, this amino group may be converted into a different functional group suitable for reaction with a cargo moiety or a linker. Thus, e.g. reaction of the amino group with succinic anhydride will provide a selectively addressable carboxyl group, while further peptide chain extension with a cysteine derivative will result in a selectively addressable thiol group. Once a suitable selectively addressable functional group has been obtained in the delivery vector precursor, a cargo moiety or a derivative thereof may be attached through e.g. amide, ester, or disulphide bond formation. Alternatively, a linker group, e.g. *m*-maleimidobenzoyl, is introduced by reaction of a linker group precursor with the selectively addressable function of the delivery vector precursor, followed by formation of a covalent bond between the linker group and a cargo moiety. Multivalent cargo-delivery vector constructs may be obtained, *inter alia*, by successive extension of the selectively addressable delivery vector precursor with trivalent chemical groups. Thus peptide chain extension with e.g. N^{α,ε}-Fmoc-protected Lys derivatives will afford di-, tetra-, and octa-valent construct precursors after one, two, or three coupling/Fmoc-deprotection cycles.

Using these methods, the skilled person will be capable of preparing a wide variety of cargo-carrier conjugates utilising a variety of linker moieties. As exemplified below, an appropriate group on the cargo moiety may be selected for attachment to the carrier moiety and if desired a linker joined to the cargo or carrier moiety, or both prior to their coupling.

The compounds of the present invention may be formulated with a physiologically acceptable diluent or carrier for use as pharmaceuticals for both veterinary, for example in mammals, and particularly human use by a variety of methods. For instance, they may be applied as a composition incorporating a liquid

diluent or carrier, for example an aqueous or oily solution, suspension or emulsion, which may often be employed in injectable form for parental administration and therefore may conveniently be sterile and pyrogen free. Oral administration may also be used and although compositions for this purpose may incorporate a liquid diluent or carrier, it is more usual to use a solid, for example a conventional solid carrier material such as starch, lactose, dextrin or magnesium stearate. Such solid compositions may take the form of powders but are more conveniently of a formed type, for example as tablets, cachets, or capsules (including spansules). Alternative, more specialized types of formulation include liposomes and nanoparticles.

Other types of administration than by injection or through the oral route which are of use in both human and veterinary contexts include the use of suppositories or pessaries. Another form of pharmaceutical composition is one for buccal or nasal administration or administration to the airways such as alveolar tissue. Other formulations of topical administration include lotions, ointments, creams, gels and sprays.

Compositions may be formulated in unit dosage form, i.e. in the form of discrete portions containing a unit dose, or a multiple or sub-unit of a unit dose.

The translocation vectors of the present invention provides several advantages over known delivery systems. These advantages include improved efficacy compared to conventional treatments, improved cellular uptake of the therapeutic agent, improved water solubility, reduction of side effects and cellular bioavailability and decreased occurrence of drug resistance.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application, as well as the Figures and the Sequence Listing are incorporated herein by reference.

EXAMPLES

5 **Example 1; Preparation of a series of peptides (peptides 1 - 20), being truncated forms of penetratin (SEQ ID No. 1).**

Abbreviations.

10 Amino acid and peptide nomenclature conforms to IUPAC-IUB rules (*Eur. J. Biochem.* 1984, **138**, 9-37). Other abbreviations: Ahx, 6-aminohexanoyl; APase, alkaline phosphatase. DE MALDI-TOF MS, delayed-extraction matrix-assisted laser desorption ionisation time-of-flight mass spectrometry. DIEA, *N,N*-diisopropylethylamine. PBS, phosphate-buffered saline (10 mM phosphate, 150 mM NaCl, pH 7.4); PyBOP, Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium
15 hexafluorophosphate; RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid.

1.1: Materials and Methods

20 *General*

The peptide deprotection / cleavage mixture used throughout was as follows: 0.75 : 0.5 : 0.5 : 0.25 : 10 (w / v / v / v / v) PhOH, H₂O, PhSMe, 1,2-ethanedithiol, TFA (Beavis, R.C. et al., (1992) *Organic Mass Spectrometry* 27, 156-158). Analytical and preparative RP-HPLC was performed using Vydac 218TP54 (4.6 x 250 mm) and
25 218TP1022 (22 x 250 mm) columns, respectively. Flow rates of 1 mL/min for analytical runs and 9 mL/min for preparative work were used (at 25 °C). Gradient elution with increasing amounts of MeCN in H₂O (containing 0.1 % TFA) over 20 min (anal.) and 40 min (prep.) was performed. Eluants were monitored at $\lambda = 200 - 300$ nm. Peptide samples were also analysed by DE MALDI-TOF mass spectrometry
30 (ThermoBioAnalysis Dynamo instrument). An α -cyano-4-hydroxycinnamic acid matrix (Beavis, R.C. et al., (1992) *Organic Mass Spectrometry* 27, 156-158) was used

and the appropriate m/z range was calibrated using authentic peptide standards in the m/z range 1,000 – 2,600.

1.2: Simultaneous multiple synthesis of peptides 1 - 20

5 Peptides were synthesised using a Multipin Peptide Synthesis Kit (Chiron Technologies Pty. Ltd., Clayton, VIC, Australia). Peptide chains were assembled on “Macro Crowns” (SynPhase HM Series I, Rink Amide Linker; 5.3 $\mu\text{mol/crown}$) using Fmoc-amino acids (100 mM in DMF) and PyBOP / HOBt / DIEA (1 : 1 : 1.5) coupling chemistry. The amino acid side-chain protecting groups were 2,2,5,7,8-
10 pentamethylchroman-6-sulphonyl (Arg), trityl (Asn and Gln), and *t*-butyloxycarbonyl (Lys and Trp). Activated amino acid solutions were dispensed using a PinAID device (Chiron Technologies). Coupling reactions were allowed to proceed for a minimum of 4 h. All other chain assembly manipulations, including repetitive deprotection reactions (20 % piperidine in DMF) and washing cycles (DMF and MeOH), were
15 carried out according to procedures set out in the kit manual. After coupling and deprotection of the *N*-terminal βAla residues, (+)-biotin (300 mM in DMF) was coupled (chemistry as above for amino acids) during 4 h. After washing and drying, the “Macro Crowns” were removed from the synthesis device and placed into 10-mL capped polypropylene tubes. To each tube was added 1.5 mL of cleavage /
20 deprotection mixture. After 2 h, the “Macro Crowns” were removed and washed with 0.5 mL each of neat TFA. To each tube containing the combined cleavage mixtures and washings Et_2O (8 mL) was added. After cooling to 4 °C, the precipitated peptides were collected by centrifugation (4 min at 5,000 r.p.m.) and decantation. The pellets were resuspended in Et_2O (5 mL / tube). The suspensions were again cooled and the
25 peptides isolated as before. The washing process was repeated once more before the crude peptides were dried *in vacuo*.

The crude peptides were redissolved in 0.1 % aq TFA using sonication (2 mL / sample) and were applied to primed (MeOH then 0.1 % aq TFA) solid-phase
30 extraction cartridges (Merck LiChrolut RP-18. 500 mg). These were successively

[illegible]

TABLE 1

N°	Peptide
1	Biotinyl-βAla-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH ₂
2	Biotinyl-βAla-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-NH ₂
3	Biotinyl-βAla-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-NH ₂
4	Biotinyl-βAla-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-NH ₂
5	Biotinyl-βAla-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-NH ₂
6	Biotinyl-βAla-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-NH ₂
7	Biotinyl-βAla-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-NH ₂
8	Biotinyl-βAla-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-NH ₂
9	Biotinyl-βAla-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-NH ₂
10	Biotinyl-βAla-Arg-Gln-Ile-Lys-Ile-Trp-NH ₂
11	Biotinyl-βAla-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH ₂
12	Biotinyl-βAla-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH ₂
13	Biotinyl-βAla-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH ₂
14	Biotinyl-βAla-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH ₂
15	Biotinyl-βAla-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH ₂
16	Biotinyl-βAla-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH ₂
17	Biotinyl-βAla-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH ₂
18	Biotinyl-βAla-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH ₂
19	Biotinyl-βAla-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH ₂
20	Biotinyl-βAla-Arg-Met-Lys-Trp-Lys-Lys-NH ₂

Ins
Bio

[illegible]

TABLE 2

Chromatographic and mass spectrometric analysis of peptides 1 - 20

N°	Formula	M _r	MS ^a	Yield			Anal. RP-HPLC	
				mg ^b	μmol	% ^c	t _R (min) ^d	Purity (%) ^e
1	C ₁₁₇ H ₁₈₈ N ₃₈ O ₂₂ S ₂	2543.12	2544.1	6.7	2.6	50	19.0	78
2	C ₁₁₁ H ₁₇₆ N ₃₆ O ₂₁ S ₂	2414.95	2416.0	6.8	2.8	53	19.4	78
3	C ₁₀₅ H ₁₆₄ N ₃₄ O ₂₀ S ₂	2286.77	2287.8	4.8	2.1	40	20.3	79
4	C ₉₄ H ₁₅₄ N ₃₂ O ₁₉ S ₂	2100.56	2101.6	7.5	3.6	67	18.5	75
5	C ₈₈ H ₁₄₂ N ₃₀ O ₁₈ S ₂	1972.39	1973.4	6.5	3.3	62	19.1	81
6	C ₈₃ H ₁₃₃ N ₂₉ O ₁₇ S	1841.20	1842.2	5.6	3.1	58	18.5	98
7	C ₇₇ H ₁₂₁ N ₂₅ O ₁₆ S	1685.01	1686.0	7.0	4.2	78	19.0	95
8	C ₇₁ H ₁₀₉ N ₂₁ O ₁₅ S	1528.82	1529.8	4.8	3.1	59	19.7	95
9	C ₆₇ H ₁₀₃ N ₁₉ O ₁₃ S	1414.72	1415.7	4.8	3.4	64	20.2	90
10	C ₅₃ H ₈₆ N ₁₆ O ₁₀ S	1139.42	1140.4	3.2	2.8	53	17.9	93
11	C ₁₁₁ H ₁₇₆ N ₃₄ O ₂₁ S ₂	2386.93	2387.9	5.1	2.1	40	19.5	82
12	C ₁₀₆ H ₁₆₈ N ₃₂ O ₁₉ S ₂	2258.80	2259.8	5.0	2.2	42	19.8	85
13	C ₁₀₀ H ₁₅₇ N ₃₁ O ₁₈ S ₂	2145.65	2146.7	5.8	2.7	51	17.6	90
14	C ₉₄ H ₁₄₅ N ₂₉ O ₁₇ S ₂	2017.47	2018.5	6.6	3.3	62	19.1	87
15	C ₈₈ H ₁₃₄ N ₂₈ O ₁₆ S ₂	1904.32	1905.3	5.3	2.8	53	17.9	90
16	C ₇₇ H ₁₂₄ N ₂₆ O ₁₅ S ₂	1718.11	1719.1	5.3	3.1	58	14.9	91
17	C ₆₈ H ₁₁₅ N ₂₅ O ₁₄ S ₂	1570.93	1571.9	5.6	3.6	67	12.2	93
18	C ₆₃ H ₁₀₇ N ₂₃ O ₁₂ S ₂	1442.80	1443.8	4.4	3.0	57	12.4	93
19	C ₅₉ H ₁₀₁ N ₂₁ O ₁₀ S ₂	1328.70	1329.7	5.1	3.9	73	12.7	94
20	C ₅₃ H ₈₉ N ₁₇ O ₉ S ₂	1172.51	1173.5	5.0	4.3	81	13.2	96

5 ^aBy DE MALDI-TOF MS.^bAfter solid-phase extraction and vacuum centrifugation.^cRelative to 5.3 μmol loading of synthesis 'crowns'.^dGradient 5 - 35 % (peptides 1 - 20) in 0.1 % aq TFA over 20 min.^eFrom chromatogram integration (λ = 214 nm).

Preparation of reduced linear and oxidised cyclic peptides 37 and 38

The peptide sequence was assembled on Fmoc-Cys(Trt)-resin (*p*-hydroxymethylphenoxyacetic acid handle, 0.50 mmol/g functionality, 0.50 g, 0.25 mmol; ABI 401418) using an ABI 433A Peptide Synthesizer (Perkin-Elmer Applied Biosystems) and standard "0.25 mmol FastMoc MonPrevPc" chemistry. After final
5 Fmoc-deprotection and washing (Et₂O), the peptidyl resin was dried *in vacuo* (1.43 g, 91 %). An aliquot (285 mg, *ca.* 0.05 mmol) of this material was resuspended in DMF, drained, and reacted with biotinamidocaproate *N*-hydroxysuccinimide ester (137 mg, 0.3 mmol), HOBt (50 mg, 0.3 mmol) and DIEA (0.14 mL 0.8 mmol) in DMF (3 mL)
10 for 18 h under N₂. The resin was then washed successively with DMF, CH₂Cl₂ and Et₂O, before being dried *in vacuo*.

The above biotinylated peptidyl resin (290 mg, *ca.* 0.05 mmol) was treated with cleavage / deprotection mixture (5 mL) for 2.5 h. Resin residue was then filtered
15 off. The filtrate was treated with Et₂O (45 mL), the mixture was cooled and the precipitated peptide was collected by centrifugation (2 min at 4,000 r.p.m.). The crude biotinylated peptide (141 mg, *ca.* quant.) was washed twice more with Et₂O in a similar manner before being dried *in vacuo*. A sample (20 mg) of this material was dissolved in 0.1 % aq TFA (2 mL), the solution was filtered and fractionated by prep.
20 RP-HPLC. Fractions containing pure material (by anal. RP-HPLC) were pooled and lyophilised to afford pure peptide **37** (12.1 mg). Anal. RP-HPLC: t_R = 20.8 min, purity > 99 % at λ = 214 nm (20 – 30 % MeCN gradient). DE MALDI-TOF MS: $[M + H]^+ = 2776$, $[2 M + H]^{2+} = 1389$ ($C_{127}H_{205}N_{39}O_{25}S_3 = 2774.43$).

Crude peptide **37** (before prep. RP-HPLC, 35 mg) was dissolved in aq
25 NH₄HCO₃ solution (0.1 M, 70 mL). The uncapped mixture was stirred for 18 h at room temperature. The resulting suspension was then acidified to pH 4 with AcOH (*ca.* 2 mL) to yield a clear solution which was evaporated to dryness by vacuum centrifugation for 18 h. The residue was redissolved in 0.1 % aq TFA (2 mL) and
30 purified by prep. RP-HPLC in a similar manner to the above reduced precursor **37**

except that the gradient was developed from 20 - 30 % MeCN. After lyophilisation, pure peptide **38** (4.5 mg) was obtained. Anal. RP-HPLC: t_R = 15.7 min, purity > 99 % at λ = 214 nm (20 - 30 % gradient). DE MALDI-TOF MS: $[M + H]^+ = 2774$, $[2 M + H]^{2+} = 1388$ ($C_{127}H_{203}N_{39}O_{25}S_3 = 2772.42$).

5

Preparation of Fluorescein-labelled Penetratin 39

The sequence was assembled in a similar fashion as described for peptide **37**, except that Fmoc-Lys(Boc)-Resin (0.5 mmol/g loading; ABI 401425) was used. The H- β Ala-Arg(Pmc)-Gln(Trt)-Ile-Lys(Boc)-Ile-Trp-Phe-Gln(Trt)-Asn(Trt)-Arg(Pmc)-Arg(Pmc)-Met-Lys(Boc)-Trp-Lys(Boc)-Lys(Boc)-Resin (300 mg, *ca.* 0.055 mmol) was reacted with 5-carboxyfluorescein (103 mg, 0.27 mmol; Sigma C 0537), PyBOP (142 mg, 0.27 mmol), HOBt (37 mg, 0.27 mmol), and DIEA (71 mL, 0.41 μ mol) in DMF (5mL) under N_2 and in the dark during 18 h. It was then washed (DMF, CH_2Cl_2 , and Et_2O) and dried *in vacuo*. After treatment during 2 h with cleavage / deprotection mixture (12 mL) in the dark and work-up as above, crude peptide was obtained (183 mg). An aliquot (90 mg) was purified by preparative RP-HPLC to afford pure peptide after lyophilisation (38 mg). Anal. RP-HPLC: t_R = 15.7 min, purity > 99 % at λ = 214 nm (22.5 - 32.5 % gradient). DE MALDI-TOF MS: $[M + H]^+ = 2677$, $[2 M + H]^{2+} = 5359$ ($C_{128}H_{183}N_{35}O_{27}S = 2676.11$).

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1.3: Peptide internalisation assay

HaCaT cells (immortalised 'normal' human fibroblast cell line) were seeded into 96-well plates at 50,000 cells per well in medium (DMEM) with 10 % foetal calf serum and antibiotics. After an overnight incubation, peptides were prepared as dilution series in cell medium and were added to the cells. At the end of the incubation period (usually 10 and 60 min), the cells were rinsed three times with PBS and fixed for 20 min at $-20^\circ C$ in EtOH / AcOH (95 : 5). After the fixation, the cells were made permeable by treatment for 10 min with PBS containing 3 % Tween-20. Endogenous alkaline phosphatase activity was neutralised by incubation at $65^\circ C$ for 60 min. Cells were incubated for 30 min at room temperature with alkaline phosphatase-streptavidin

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(Pierce Chemical Co., Rockford, IL, USA) in 0.1 % BSA in PBS and washed extensively with PBS. Freshly prepared substrate solution (1 mg/mL *p*-nitrophenyl phosphate disodium (Pierce Chemical Co.) in 10 mM diethanolamine (pH 9.5) containing 0.5 mM MgCl₂ was added to each well and incubated until sufficient colour
5 had developed (approximately 30 min.). The enzymatic reaction was stopped by adding 50 µL 2 M aq NaOH to each well. Alkaline phosphatase activity was measured spectrophotometrically at 405 nm.

10 1.4: Results

1.4.1: Peptide synthesis

Peptides **1 – 20** were prepared simultaneously using the so-called Multipin™ method (Valerio, R.M. et al., (1993) International Journal of Peptide and Protein Research 42, 1-9). On average 2 coupling/deprotection cycles were performed per day
15 and the entire synthesis, including biotinylation, cleavage / deprotection, purification by solid phase extraction, and analysis, were completed within a fortnight. The isolated and purified yields of the peptides ranged from 40 – 81 % and purities were 75 – 96 % (Table 2). The excellent quality of the peptides is demonstrated in Figure 1. The main impurities observed (MS, data not shown) were Met(O)-containing peptides (leading
20 peaks on traces in Fig. 1. Methionine sulfoxide formation appears to be a general problem attendant in the Multipin method, presumably due to oxidation during the extended air drying cycles after the acylation and deprotection steps. In principle it is possible to back-reduce Met(O) in peptides; *e.g.*, on an analytical scale we were able to convert the Met(O)-containing impurity to **1** cleanly using NH₄I / Me₂S in TFA
25 (Nicolás, E et al., (1995) Tetrahedron 51, 57013) (results not shown).

1.4.2: Determination of minimal active sequence

As discussed above, the 16mer peptide subtending residues 43 – 58 of the Antennapedia homeodomain was originally identified as the minimal sequence
30 retaining efficient translocation properties using peptides corresponding to residues 41

- 60, 43 -58, 41 - 55, and 46 – 60. The results (*Figure 2*) show that truncation at the C-terminus of peptide **1** results in a reduction in the degree of internalisation but nevertheless, the truncated peptides were still capable of translocating the cell membrane. Successive truncation from the *N*-terminus of peptide **1**, however shows
5 that significant levels of translocation are retained in the truncated peptides in many cases approaching the level attained by peptide **1** itself. Little activity was lost upon the first three truncations (**11** - **13**) but only about half of the original signal was left with derivatives **14** and **15**. However, at the 10mer to 7mer (**16** – **19**) stage almost full membrane translocation efficiency relative to control peptide **1** was regained before a
10 severe drop was observed with the C-terminal 6mer peptide **20**. This effect was reproducible in several independent experiments. While values for only one peptide concentration are shown in *Figure 2*, dose-response curves for each individual peptide have shown that the same pattern was seen, regardless of peptide concentration. Non-specific binding in the absence of cells has been found to be uniform and negligible
15 (results not shown).

1.4.3 Peptide internalisation assay of fluorescein-labelled Penetratin 39

This assay permitted the study and measurement of cell internalisation without the possibility of observing artefacts emanating from the cell manipulations necessary
20 with the biotinylated peptides. *Figure 4* shows the direct measurement of peptide internalisation into live cells. (squares when t=10 mins, diamonds when t=60 mins). As can be seen in *Figure 4*, biotinylated Penetratin **1** localises predominantly to the cell nucleus and accumulates in the nucleoli, with lesser concentration in the cytosol. Clearly the distribution is very similar when the direct fluorescent probe **39** is used,
25 thus validating the indirect biotin – avidin visualisation approach. The fact that Penetratin appears to localise mainly to the nucleus shows that this peptide can in fact translocate across both plasma and nuclear membranes. Nucleolar accumulation may be due to non-specific binding of the positively charged peptide to DNA.

2.1 In accordance with the methods described in sections 1.1 and 1.2 above, the following peptides were prepared;

KKWKORR.

Each of these peptides were used in the peptide internalisation assay described in section 1.3 above and was found to be internalised into cells.

25

INS
B12

2000 2001 2002 2003 2004 2005 2006 2007 2008 2009 2010 2011 2012 2013 2014 2015 2016 2017 2018 2019 2020 2021 2022 2023 2024 2025 2026 2027 2028 2029 2030 2031 2032 2033 2034 2035 2036 2037 2038 2039 2040 2041 2042 2043 2044 2045 2046 2047 2048 2049 2050 2051 2052 2053 2054 2055 2056 2057 2058 2059 2060 2061 2062 2063 2064 2065 2066 2067 2068 2069 2070 2071 2072 2073 2074 2075 2076 2077 2078 2079 2080 2081 2082 2083 2084 2085 2086 2087 2088 2089 2090 2091 2092 2093 2094 2095 2096 2097 2098 2099 2100 2101 2102 2103 2104 2105 2106 2107 2108 2109 2110 2111 2112 2113 2114 2115 2116 2117 2118 2119 2120 2121 2122 2123 2124 2125 2126 2127 2128 2129 2130 2131 2132 2133 2134 2135 2136 2137 2138 2139 2140 2141 2142 2143 2144 2145 2146 2147 2148 2149 2150 2151 2152 2153 2154 2155 2156 2157 2158 2159 2160 2161 2162 2163 2164 2165 2166 2167 2168 2169 2170 2171 2172 2173 2174 2175 2176 2177 2178 2179 2180 2181 2182 2183 2184 2185 2186 2187 2188 2189 2190 2191 2192 2193 2194 2195 2196 2197 2198 2199 2200 2201 2202 2203 2204 2205 2206 2207 2208 2209 2210 2211 2212 2213 2214 2215 2216 2217 2218 2219 2220 2221 2222 2223 2224 2225 2226 2227 2228 2229 2230 2231 2232 2233 2234 2235 2236 2237 2238 2239 2240 2241 2242 2243 2244 2245 2246 2247 2248 2249 2250 2251 2252 2253 2254 2255 2256 2257 2258 2259 2260 2261 2262 2263 2264 2265 2266 2267 2268 2269 2270 2271 2272 2273 2274 2275 2276 2277 2278 2279 2280 2281 2282 2283 2284 2285 2286 2287 2288 2289 2290 2291 2292 2293 2294 2295 2296 2297 2298 2299 2300 2301 2302 2303 2304 2305 2306 2307 2308 2309 2310 2311 2312 2313 2314 2315 2316 2317 2318 2319 2320 2321 2322 2323 2324 2325 2326 2327 2328 2329 2330 2331 2332 2333 2334 2335 2336 2337 2338 2339 2340 2341 2342 2343 2344 2345 2346 2347 2348 2349 2350 2351 2352 2353 2354 2355 2356 2357 2358 2359 2360 2361 2362 2363 2364 2365 2366 2367 2368 2369 2370 2371 2372 2373 2374 2375 2376 2377 2378 2379 2380 2381 2382 2383 2384 2385 2386 2387 2388 2389 2390 2391 2392 2393 2394 2395 2396 2397 2398 2399 2400 2401 2402 2403 2404 2405 2406 2407 2408 2409 2410 2411 2412 2413 2414 2415 2416 2417 2418 2419 2420 2421 2422 2423 2424 2425 2426 2427 2428 2429 2430 2431 2432 2433 2434 2435 2436 2437 2438 2439 2440 2441 2442 2443 2444 2445 2446 2447 2448 2449 2450 2451 2452 2453 2454 2455 2456 2457 2458 2459 2460 2461 2462 2463 2464 2465 2466 2467 2468 2469 2470 2471 2472 2473 2474 2475 2476 2477 2478 2479 2480 2481 2482 2483 2484 2485 2486 2487 2488 2489 2490 2491 2492 2493 2494 2495 2496 2497 2498 2499 2500 2501 2502 2503 2504 2505 2506 2507 2508 2509 2510 2511 2512 2513 2514 2515 2516 2517 2518 2519 2520 2521 2522 2523 2524 2525 2526 2527 2528 2529 2530 2531 2532 2533 2534 2535 2536 2537 2538 2539 2540 2541 2542 2543 2544 2545 2546 2547 2548 2549 2550 2551 2552 2553 2554 2555 2556 2557 2558 2559 2560 2561 2562 2563 2564 2565 2566 2567 2568 2569 2570 2571 2572 2573 2574 2575 2576 2577 2578 2579 2580 2581 2582 2583 2584 2585 2586 2587 2588 2589 2590 2591 2592 2593 2594 2595 2596 2597 2598 2599 2600 2601 2602 2603 2604 2605 2606 2607 2608 2609 2610 2611 2612 2613 2614 2615 2616 2617 2618 2619 2620 2621 2622 2623 2624 2625 2626 2627 2628 2629 2630 2631 2632 2633 2634 2635 2636 2637 2638 2639 2640 2641 2642 2643 2644 2645 2646 2647 2648 2649 2650 2651 2652 2653 2654 2655 2656 2657 2658 2659 2660 2661 2662 2663 2664 2665 2666 2667 2668 2669 2670 2671 2672 2673 2674 2675 2676 2677 2678 2679 2680 2681 2682 2683 2684 2685 2686 2687 2688 2689 2690 2691 2692 2693 2694 2695 2696 2697 2698 2699 2700 2701 2702 2703 2704 2705 2706 2707 2708 2709 2710 2711 2712 2713 2714 2715 2716 2717 2718 2719 2720 2721 2722 2723 2724 2725 2726 2727 2728 2729 2730 2731 2732 2733 2734 2735 2736 2737 2738 2739 2740 2741 2742 2743 2744 2745 2746 2747 2748 2749 2750 2751 2752 2753 2754 2755 2756 2757 2758 2759 2760 2761 2762 2763 2764 2765 2766 2767 2768 2769 2770 2771 2772 2773 2774 2775 2776 2777 2778 2779 2780 2781 2782 2783 2784 2785 2786 2787 2788 2789 2790 2791 2792 2793 2794 2795 2796 2797 2798 2799 2800 2801 2802 2803 2804 2805 2806 2807 2808 2809 2810 2811 2812 2813 2814 2815 2816 2817 2818

5 **3.1: Simultaneous multiple synthesis of peptides 21 - 36**

TABLE 3

N°	Peptide
1	Biotinyl-βAla-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH ₂
21	Biotinyl-βAla-Ala-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH ₂
22	Biotinyl-βAla-Arg-Ala-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH ₂
23	Biotinyl-βAla-Arg-Gln-Ala-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH ₂
24	Biotinyl-βAla-Arg-Gln-Ile-Ala-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH ₂
25	Biotinyl-βAla-Arg-Gln-Ile-Lys-Ala-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH ₂
26	Biotinyl-βAla-Arg-Gln-Ile-Lys-Ile-Ala-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH ₂
27	Biotinyl-βAla-Arg-Gln-Ile-Lys-Ile-Trp-Ala-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH ₂
28	Biotinyl-βAla-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Ala-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH ₂
29	Biotinyl-βAla-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Ala-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH ₂
30	Biotinyl-βAla-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Ala-Arg-Met-Lys-Trp-Lys-Lys-NH ₂
31	Biotinyl-βAla-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Ala-Met-Lys-Trp-Lys-Lys-NH ₂
32	Biotinyl-βAla-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Ala-Lys-Trp-Lys-Lys-NH ₂
33	Biotinyl-βAla-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Ala-Trp-Lys-Lys-NH ₂
34	Biotinyl-βAla-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Ala-Lys-Lys-NH ₂
35	Biotinyl-βAla-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Ala-Lys-NH ₂
36	Biotinyl-βAla-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Ala-NH ₂

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TABLE 4

Chromatographic and mass spectrometric analysis of peptides 1 and 21- 36

N°	Formula	M _r	MS ^a	Yield			Anal. RP-HPLC	
				mg ^b	μmo l	% ^c	t _R (min) ^d	Purity (%) ^e
1	C ₁₁₇ H ₁₈₈ N ₃₈ O ₂₂ S ₂	2543.12	2544.1	6.7	2.6	50	19.0	78
21	C ₁₁₄ H ₁₈₁ N ₃₅ O ₁₁ S ₂	2458.01	2459.0	5.7	2.3	44	16.8	66
22	C ₁₁₅ H ₁₈₅ N ₃₇ O ₂₁ S ₂	2486.00	2487.1	6.2	2.5	47	16.9	79
23	C ₁₁₄ H ₁₈₂ N ₃₈ O ₂₂ S ₂	2501.04	2502.0	5.8	2.3	44	12.9	80
24	C ₁₁₄ H ₁₈₁ N ₃₇ O ₂₂ S ₂	2486.00	2487.0	6.4	2.6	48	17.6	76
25	C ₁₁₄ H ₁₈₂ N ₃₈ O ₂₂ S ₂	2501.04	2502.0	6.8	2.7	51	15.7	80
26	C ₁₀₉ H ₁₈₃ N ₃₇ O ₂₂ S ₂	2427.99	2429.0	6.7	2.7	52	12.5	80
27	C ₁₁₁ H ₁₈₄ N ₃₈ O ₂₂ S ₂	2467.02	2468.0	6.1	2.5	47	12.8	78
28	C ₁₁₅ H ₁₈₅ N ₃₇ O ₂₁ S ₂	2486.07	2487.1	6.5	2.6	49	15.9	79
29	C ₁₁₆ H ₁₈₇ N ₃₇ O ₂₁ S ₂	2500.09	2501.1	5.7	2.3	43	16.2	75
30	C ₁₁₄ H ₁₈₁ N ₃₅ O ₂₂ S ₂	2458.01	2459.0	5.6	2.3	43	18.7	77
31	C ₁₁₄ H ₁₈₁ N ₃₅ O ₂₂ S ₂	2458.01	2459.0	7.5	3.1	58	16.8	77
32	C ₁₁₅ H ₁₈₄ N ₃₈ O ₂₂ S ₂	2483.00	2484.0	6.4	2.6	49	15.4	96
33	C ₁₁₄ H ₁₈₁ N ₃₇ O ₂₂ S ₂	2486.03	2487.0	6.8	2.7	52	16.6	79
34	C ₁₀₉ H ₁₈₃ N ₃₇ O ₂₂ S ₂	2427.99	2429.0	8.5	3.5	66	14.3	83
35	C ₁₁₄ H ₁₈₁ N ₃₇ O ₂₂ S ₂	2486.03	2487.0	9.0	3.6	68	16.5	78
36	C ₁₁₄ H ₁₈₁ N ₃₇ O ₂₂ S ₂	2486.03	2487.0	10.1	4.0	76	16.4	73

^aBy DE MALDI-TOF MS.5 ^bAfter solid-phase extraction and vacuum centrifugation.^cRelative to 5.3 μmol loading of synthesis 'crowns'.^dGradient 15 - 35 % acetonitrile (peptides **21** - **36**) in 0.1 % aq TFA over 20 min.^eFrom chromatogram integration (λ = 214 nm).10 **3.2** Peptide Internalisation assays were performed in accordance with section 1.3.**3.3: Results****3.3.1: Peptide synthesis**15 Peptides **21** – **36** were prepared simultaneously using the so-called Multipin™ method (Valerio, R.M.et al., (1993) International Journal of Peptide and Protein

Research 42, 1-9). On average 2 coupling/deprotection cycles were performed per day and the entire synthesis, including biotinylation, cleavage / deprotection, purification by solid phase extraction, and analysis, were completed within a fortnight. The isolated and purified yields of the peptides ranged from 43 – 76 % and purities were 66 – 96 % (Table 2). The main impurities observed (MS, data not shown) were Met(O)-containing peptides. Methionine sulfoxide formation appears to be a general problem attendant in the Multipin method, presumably due to oxidation during the extended air drying cycles after the acylation and deprotection steps. In principle it is possible to back-reduce Met(O) in peptides; e.g., on an analytical scale we were able to convert the Met(O)-containing impurity to **1** cleanly using NH_4I / Me_2S in TFA (Nicolás, E et al., (1995) Tetrahedron 51, 57013) (results not shown).

3.3.2: Effect of residue substitutions

The results using a set of peptides in which each residue in turn had been substituted with Ala (peptides **21** – **36**) are shown in Figure 5. The results clearly show that there are no very stringent requirements for any particular hydrophobic residue. It was shown elsewhere that e.g. both Ile residues can be substituted with Val, apparently without loss of activity (Brugidou, J et al., (1995) Biochemical and Biophysical Research Communications 214, 685-693). Furthermore, Met¹² is freely exchangeable with either Leu or Nle (results not shown). What these results clearly demonstrate is that in contrast to the prevalent opinion in the prior art, there is no necessity for the Trp⁶.

Example 4; Further modified penetratin derivatives

Using the method described in sections 1.1 and 1.2 above, further modified peptides of SEQ ID No. 1 may be prepared replacing the alanine residue shown in bold with any other amino acid residue, including the following peptides which were all active in the cell internalisation assay described in section 1.3 above;

Modification to Penetratin*	Sequence
Met55 Nle	RQIKIWFQNRROKWKK
Met55 Nle (retro)	KKWKORRNQFWIKIQR
Gln50Pro	RQIKIWFPNRRMKWKK
45,50,55Pro	RQPIKIWFNRRMPWKK
Trp48,56Phe	RQIKIFFQNRNRMKFKK

* numbering refers to the corresponding residues as they appear in Anntenapedia, where penetratin is denoted as AntP(43-58).

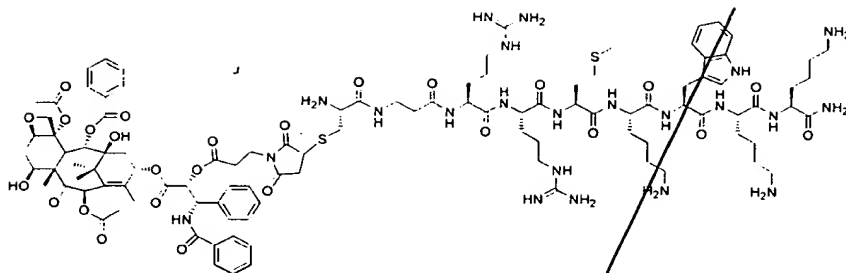
- 5 **Examples 5-17; Using the methods described above translocation vectors**
comprising the carrier moiety linked to a cargo were prepared as described.

10 Example 5

H-Cys-βAla-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH₂

Starting from Rink Amide AM resin (0.69 mmol/g, Novabiochem), H-Cys(Trt)- β Ala-Arg(Pmc)-Arg(Pmc)-Met-Lys(Boc)-Trp-Lys(Boc)-Lys(Boc)-resin was assembled. After deprotection (1.5 h), the crude peptide was obtained by precipitation from Et₂O, centrifugation/decantation, and drying. Aliquots (total 246 mg) were purified by preparative RP-HPLC (6.5 – 16.5 % MeCN gradient) to afford the pure title compound (106.4 mg). Anal. RP-HPLC: t_R = 15.8 min (6.5 – 16.5 % MeCN gradient, purity > 95 %, λ = 214 nm). DE MALDI-TOF MS: $[M + H]^+ = 1205.4$ ($C_{53}H_{92}N_{20}O_9S_2 = 1205.55$).

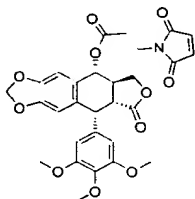
2'-[Succinimidopropionoyl-(H-Cys-βAla-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH₂)]paclitaxel



To a solution of 2'-(maleimidopropionoyl)paclitaxel (17 μmol , 17.4 mg) and
 H-Cys- β Ala-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH₂ (15 μmol , 18.1 mg) in DMF (1 mL)
 was added Et₃N (2.0 μL). The mixture was stirred for 1h, filtered and purified by
 preparative RP-HPLC (10 – 70 % MeCN gradient). The pure title compound (9.4 mg)
 was obtained as a colourless solid. Anal. RP-HPLC: t_R = 17.2 min (0 – 60 % MeCN
 gradient, purity > 97 %). DE MALDI-TOF MS: $[M + H]^+ = 2211.7$ ($\text{C}_{106}\text{H}_{148}\text{N}_{22}\text{O}_{26}\text{S}_2 =$
 2210.57.

Example 6

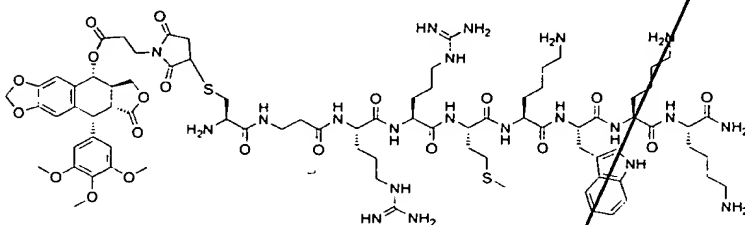
4-(Maleimidopropionoyl)podophyllotoxin



A solution of podophyllotoxin (60 μmol , 25.6 mg), 3-maleimidopropionic acid
 (0.31 mmol, 52.4 mg), DIC (0.17 mmol, 21.5 mg) and DMAP (80 μmol , 10 mg) in
 CH₂Cl₂ (2 mL) was stirred for 1 h. The solvent was evaporated *in vacuo* and the
 residue was redissolved in DMF/MeOH (1 mL) and purified by preparative RP-HPLC
 (20 – 70 % MeCN gradient) to afford the pure title compound as a colourless solid (7.3
 mg). Anal. RP-HPLC: t_R = 20.1 min (0 – 60 % MeCN gradient, purity > 95 %). ¹H-
 NMR (300 MHz, CDCl₃) δ : 2.66-2.71 (t, J = 6.3 Hz, 2H, CH₂), 2.82-2.84 (m, 2H, H2
 and H3), 3.69 (s, 6H, OCH₃x2), 3.75 (s, 3H, OCH₃), 3.83 (t, J = 6.3 Hz, 2H, CH₂), 4.12
 (t, J = 9.92 Hz, 1H, H11), 4.31 (m, 1H, H11), 4.53 (d, J = 11.4 Hz, 1H, H1), 5.80 (d, J

= 8.7 Hz, 1H, H4), 5.92 (dd, $J = 5.49, 1.17$ Hz, 2H, OCH₂O), 6.32 (s, 2H, H2'6'), 6.47 (s, 1H, H8), 6.66 (s, 2H, CH=CH), 6.74 (s, 1H, H5).

4-[Succinimidopropionoyl-(H-Cys-βAla-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH₂)]podophyllotoxin



To a solution of 4-(maleimidopropionoyl)podophyllotoxin (17.7 μmol, 10 mg) and H-Cys-βAla-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH₂ (25 μmol, 30.4 mg) in DMF (1.5 mL) was added Et₃N (3.5 μL). The mixture was stirred for 40 min, filtered and purified by preparative RP-HPLC (0 – 60 % MeCN gradient). The pure title compound was obtained as a colourless solid (17.8 mg, 57 %). Anal. RP-HPLC: $t_R = 14.8$ min (0 – 60 % MeCN gradient, purity > 98 %). DE MALDI-TOF MS: $[M + H]^+ = 1772.3$ ($C_{81}H_{119}N_{21}O_{20}S_2 = 1771.07$).

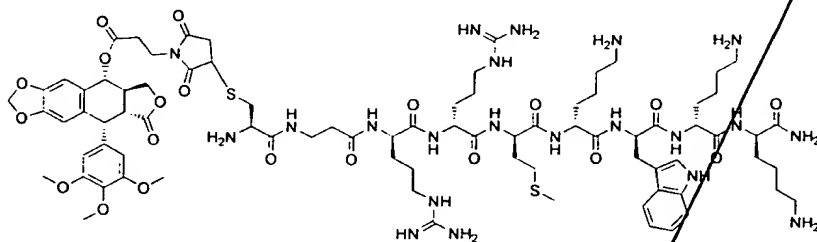
Example 7

H-Cys-βAla-D-Arg-D-Arg-D-Met-D-Lys-D-Trp-D-Lys-D-Lys-NH₂

Starting from Rink Amide AM resin (0.69 mmol/g, Novabiochem). H-Cys(Trt)-βAla-D-Arg(Pmc)-D-Arg(Pmc)-D-Met-D-Lys(Boc)-D-Trp-D-Lys(Boc)-D-Lys(Boc)-resin was assembled. After deprotection (1.5 h), the crude peptide was obtained by precipitation from Et₂O, centrifugation/decantation, and drying. Aliquots (total 237 mg) were purified by preparative RP-HPLC (8 – 18 % MeCN gradient) to afford the pure title compound (66 mg). Anal. RP-HPLC: $t_R = 12.9$ min (9 – 19 % MeCN gradient, purity > 99 %, $\lambda = 214$ nm). DE MALDI-TOF MS: $[M + H]^+ = 1207.2$ ($C_{52}H_{92}N_{20}O_9S_2 = 1205.55$).

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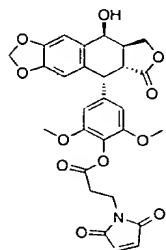
4-[Succinimidopropionoyl-(H-Cys-β-Ala-D-Arg-D-Arg-D-Met-D-Lys-D-Trp-D-Lys-D-Lys-NH₂)]podophyllotoxin



To a solution of 4-(maleimidopropionoyl)podophyllotoxin (18.9 μmol, 10.7 mg) and H-Cys-βAla-D-Arg-D-Arg-D-Met-D-Lys-D-Trp-D-Lys-D-Lys-NH₂ (28 μmol, 33.8 mg) in DMF (1.5 mL) was added Et₃N (1.5 μL). The mixture was stirred for 40 min, filtered and purified by preparative RP-HPLC (0 – 60 % MeCN gradient). The pure title compound was obtained as a colourless solid (6.9 mg, 21 %). Anal. RP-HPLC: t_R = 14.8 min (0 – 60 % MeCN gradient, purity > 98%). DE MALDI-TOF MS: [M + H]⁺ = 1771.5 (C₈₁H₁₁₉N₂₁O₂₀S₂ = 1771.07).

Example 8

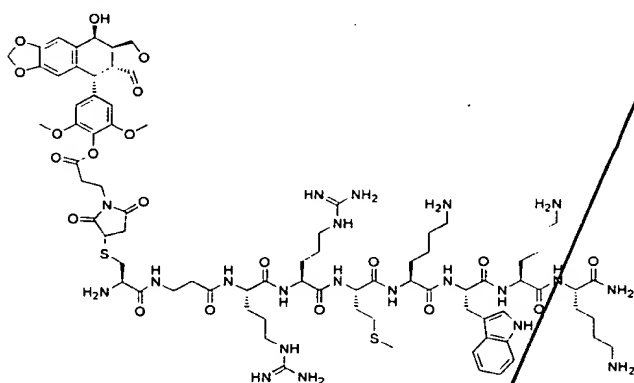
4'-(Maleimidopropionoyl)epipodophyllotoxin



A solution of 4'-demethylepipodophyllotoxin (12 mmol, 5 mg), 3-maleimidopropionic acid (50 μmol, 12.2 mg) and DIC (28 μmol, 3.47 mg) in pyridine (1 mL) was stirred for 30 min. MeOH (0.5 mL) was added and the mixture was purified by preparative RP-HPLC (0 – 60 % MeCN gradient) to afford the pure title compound as a colourless solid (4.2 mg, 62 %). Anal. RP-HPLC: t_R = 17.6 min (0 – 60 % MeCN gradient, purity > 95 %). ¹H-NMR (300 MHz, CDCl₃) δ: 2.84 (m, 1H,

H3), 2.99 (t, $J = 7.44$ Hz, 2H, $\text{CH}_2\text{-Mim}$), 3.32 (dd, $J = 14.04, 5.07$ Hz, 1H, H2), 3.69 (s, 6H, $\text{OCH}_3 \times 2$), 3.95 (t, $J = 7.44$ Hz, 2H, $\text{CH}_2\text{-Mim}$), 4.39 (dd, $J = 8.13, 4.28$ Hz, 2H, H11), 4.66 (d, $J = 5.00$ Hz, 1H, H1), 4.89 (d, $J = 3.32$ Hz, 1H, H4), 6.01 (d, $J = 6.42$ Hz, 2H, OCH_2O), 6.32 (s, 2H, H2'6'), 6.57 (s, 1H, H8), 6.74 (s, 2H, CH=CH), 6.90 (s, 1H, H5). $^{13}\text{C-NMR}$ (75 MHz, CDCl_3) δ : 28.64, 31.02, 32.55, 37.33, 39.53, 42.99, 55.15, 65.78, 66.56, 100.65, 106.54, 107.97, 109.65, 130.68, 130.92, 133.21, 136.96, 146.62, 147.61, 150.39, 167.36, 169.30, 173.89.

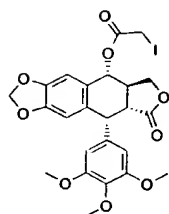
10 **4'-[Succinimidopropionoyl-(H-Cys- β Ala-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH₂)]epipodophyllotoxin**



To a solution of 4'-(maleimidopropionoyl)epipodophyllotoxin (14 μmol , 7.9 mg) and H-Cys- β Ala-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH₂ (26 μmol , 31.5 mg) in DMF (1 mL) was added Et₃N (1.9 μL). After stirring for 40 min, the mixture was purified by preparative RP-HPLC (0 – 60 % gradient) to afford the pure title compound as a colourless solid (15.8 mg, 63 %). Anal. RP-HPLC: $t_R = 13.3$ min (0 – 60 % MeCN gradient, purity > 98 %). DE MALDI-TOF MS: $[\text{M} + \text{H}]^+ = 1757.2$ ($\text{C}_{80}\text{H}_{117}\text{N}_{21}\text{O}_{20}\text{S}_2 = 1757.05$).

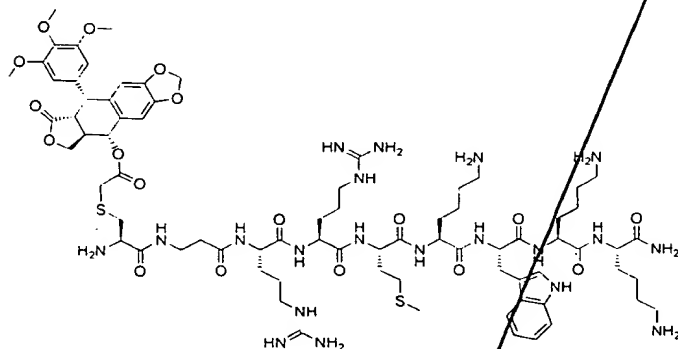
20 **Example 9**

4-(Iodoacetyl)podophyllotoxin



15

4-[Acetyl-(H-Cys-βAla-Arg-Arg-Met-Lys-Trp)₇Lys-Lys-NH₂)]podophyllotoxin



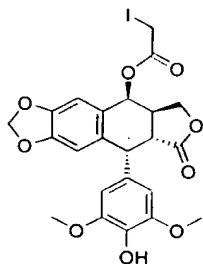
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14.1 min (0 – 60 % MeCN gradient, purity > 98 %). DE MALDI-TOF MS: $[M + H]^+ = 1661.0$ ($C_{76}H_{114}N_{20}O_{18}S_2 = 1659.97$).

5 Example 10

4'-Demethyl-4-(iodoacetyl)epipodophyllotoxin

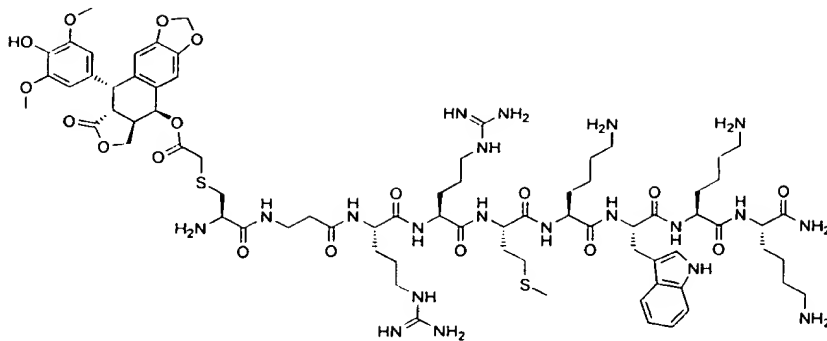


To a solution of 4'-demethylepipodophyllotoxin (0.26 mmol, 104 mg),
 10 iodoacetic acid (0.53 mmol, 98.8 mg), and DIC (0.32 mmol, 40.1 mg) in CH_2Cl_2 (2 mL) at 0 °C was added pyridine (50 μ L) and DMAP (0.1 mmol, 12.8 mg). After 1 h stirring the solvents were evaporated. The residue was redissolved in DMF (1 mL) and purified by preparative RP-HPLC (20 – 60 % MeCN gradient) to afford the pure title compound as a colourless solid (35.7 mg, 24 %). Anal. RP-HPLC: $t_R = 20.3$ min (0 –
 15 60 % MeCN gradient, purity > 96 %). 1H -NMR (300 MHz, $CDCl_3$) δ : 3.02 (m, 1H, H3), 3.20 (m, 1H, H2), 3.71 (s, 6H, $OCH_3 \times 2$), 3.63 (s, 2H, CH_2I), 3.74 (s, 3H, OCH_3), 4.05 (m, 1H, H11), 4.27 (m, 1H, H11), 4.60 (d, 1H, $J = 4.94$ Hz, H1), 6.06 (d, 1H, $J = 3.41$ Hz, H4), 5.92 (m, 2H, OCH_2O), 6.21 (s, 2H, H2'6'), 6.49 (s, 1H, H8), 6.80 (s, 1H, H5).

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4'-Demethyl-4-[acetyl-(H-Cys- β Ala-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH₂)]epipodophyllotoxin

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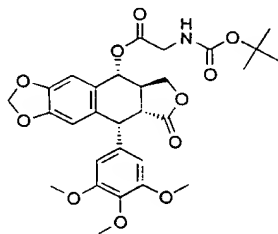


Ins B22

To a solution of 4'-demethyl-4-(iodoacetyl)epipodophyllotoxin (17.6 μmol , 10 mg) and H-Cys- β Ala-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH₂ (14.9 μmol , 18 mg) in DMF (1 mL) was added Et₃N (2.1 μL , 15 μmol). After stirring for 1 h the reaction mixture was purified by preparative RP-HPLC (0 - 60% MeCN gradient) to afford the pure title compound as a colourless solid (11.2 mg, 46 %). Anal. RP-HPLC: t_R = 12.8 min (0 - 60 % MeCN gradient, purity > 98 %). DE MALDI-TOF MS: $[M + H]^+ = 1647.2$ ($C_{75}H_{112}N_{20}O_{18}S_2 = 1645.95$).

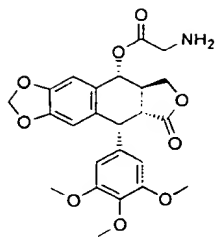
10 Example 11

4-(Boc-Gly)podophyllotoxin



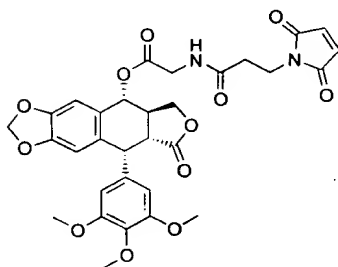
A mixture of podophyllotoxin (400 mg, 0.97 mmol), Boc-Gly-OH (510 mg, 2.91 mmol) DIC (1.73 mmol, 273 μL), DMAP (0.41 mmol, 50 mg) and pyridine (173 μL) in CH₂Cl₂ (5 mL) was stirred at for 1 h. The solvents were evaporated. The residue was redissolved in DMF (1.5 mL) and purified by RP-HPLC (20 - 70 % MeCN gradient) to afford the pure title compound as a colourless solid (502.6 mg, 91 %). Anal. RP-HPLC: t_R = 22.1 min (0 - 60 % MeCN gradient, purity > 97 %).

4-(H-Gly)podophyllotoxin



To a solution of 4-(Boc-Gly)podophyllotoxin (0.24 mmol, 137 mg) in CH_2Cl_2 (8 mL) was added TFA (0.5 mL). After stirring for 1 h the solvents were evaporated. The resulting light-yellow solid residue was purified by preparative RP-HPLC (10 – 70
5 % MeCN gradient) to afford the pure title compound as a colourless solid (41.7 mg, 37 %). Anal. RP-HPLC: t_R = 15.2 min (0 – 60 % MeCN gradient, purity > 97 %).

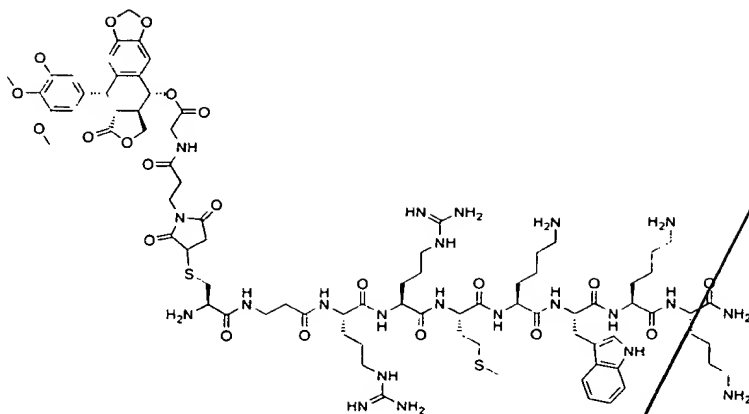
4-(Maleimidopropionoyl-Gly)podophyllotoxin



To a solution of 3-maleimidopropionic acid (70 μmol , 11.8 mg) and DIC (38
10 μmol , 4.83 mg) in DMF (1 mL) was added 4-(H-Gly)podophyllotoxin (17 μmol , 8 mg), DMAP (10 μmol , 1.2 mg) and pyridine (20 μL). After stirring for 1 h the mixture was purified by preparative RP-HPLC (0 – 60 % MeCN gradient) to afford the pure title compound as a colourless solid (1.1 mg). Anal. RP-HPLC: t_R = 18.2 min (0 – 60
15 % MeCN gradient, purity >97%).

~~4-[(Succinimidopropionoyl-Gly)-(H-Cys- β Ala-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH₂)]podophyllotoxin~~

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Ins B23

To a solution of 4-(maleimidopropionoyl-Gly)podophyllotoxin (1.8 μ mol, 1.1 mg) and H-Cys- β Ala-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH₂ (4 μ mol, 5 mg) in DMF (1 mL) was added Et₃N (0.5 μ L, 4 μ mol). The mixture was stirred for 1 h. It was diluted with MeCN (0.5 mL) and purified by preparative RP-HPLC (0 – 60 % MeCN gradient) to afford the title compound as a colourless solid (1.1 mg, 33 %). Anal. RP-HPLC: t_R = 14.7 min (0 – 60 % MeCN gradient, purity > 97 %). DE MALDI-TOF MS: $[M + H]^+ = 1829.8$ ($C_{83}H_{122}N_{22}O_{21}S_2 = 1828.12$).

10 Example 12

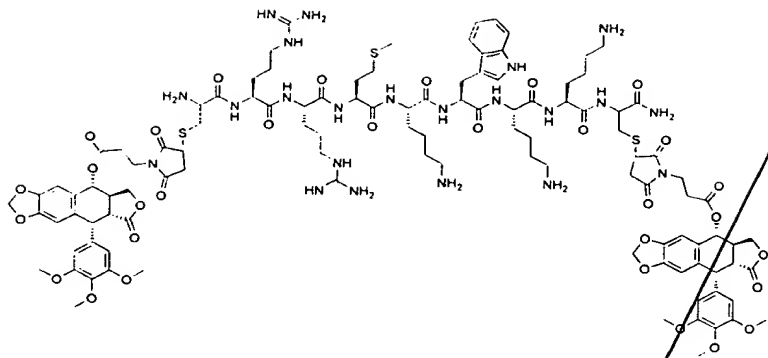
H-Cys-Arg-Arg-Met-Lys-Trp-Lys-Lys-Cys-NH₂

Ins B24

Starting from Rink Amide AM resin (0.69 mmol/g, Novabiochem), H-Cys(Trt)-Arg(Pmc)-Arg(Pmc)-Met-Lys(Boc)-Trp-Lys(Boc)-Lys(Boc)-Cys(Trt)-resin was assembled. After deprotection (1.5 h), the crude peptide was obtained by precipitation from Et₂O, centrifugation/decantation, and drying. Aliquots (total 258 mg) were purified by preparative RP-HPLC (9 – 19 % MeCN gradient) to afford the pure title compound (132.4 mg). Anal. RP-HPLC: t_R = 20.3 min (8 – 18 % MeCN gradient, purity > 99 %, $\lambda = 214$ nm). DE MALDI-TOF MS: $[M + H]^+ = 1238.6$ ($C_{52}H_{92}N_{20}O_9S_3 = 1237.63$).

Ins B23

Bis-[4-(succinimidopropionoyl)podophyllotoxin]-(H-Cys-Arg-Arg-Met-Lys-Trp-Lys-Lys-Cys-NH₂)

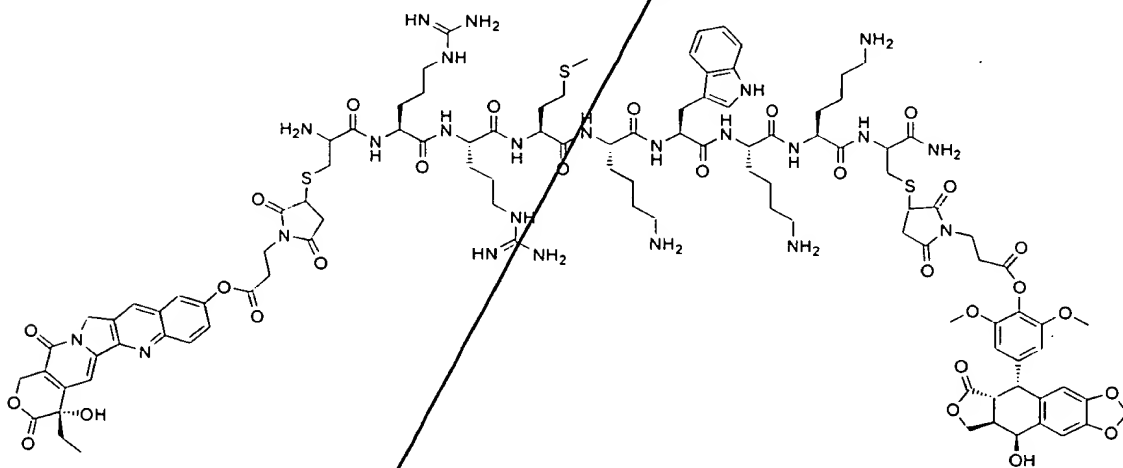


To a solution of 4-(maleimidopropionyl)podophyllotoxin (19 μmol , 11 mg) and H-Cys-Arg-Arg-Met-Lys-Trp-Lys-Lys-Cys-NH₂ (12 μmol , 15 mg), in DMF (1 mL) was added Et₃N (2.8 μL). After stirring for 1 h the mixture was purified by preparative RP-HPLC (10 – 70 % MeCN gradient) to afford the pure title compound as a colourless solid (9.0 mg, 32 %). Anal. RP-HPLC: t_R = 17.4 min (0 – 60 % MeCN gradient, purity > 98 %). DE MALDI-TOF MS: $[M + H]^+ = 2369.7$ ($C_{110}H_{146}N_{22}O_{31}S_3 = 2368.66$).

10

Example 13

4'-(Succinimidopropionyl)epipodophyllotoxin-(H-Cys-Arg-Arg-Met-Lys-Trp-Lys-Lys-Cys-NH₂)-10-O-(succinimidopropionyl)camptothecin



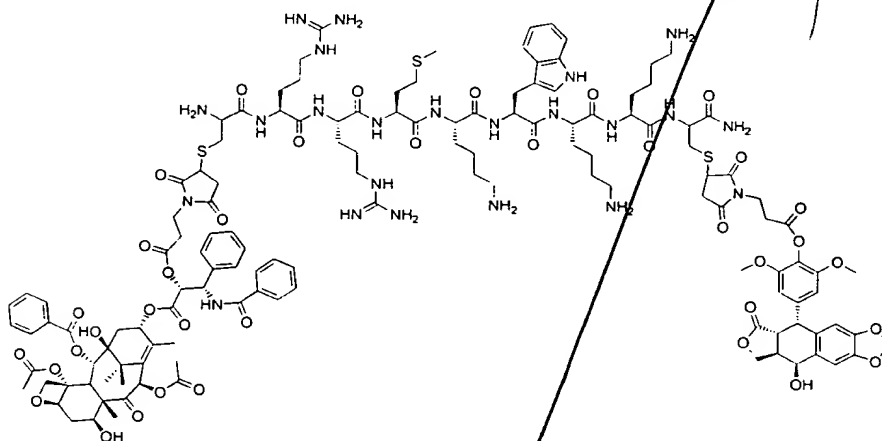
15

To a solution of 10-O-(maleimidopropionyl)camptothecin (0.005 mmol, 2.6 mg), 4'-(maleimidopropionyl) epipodophyllotoxin (5.6 μmol , 3.1 mg), and H-Cys-

Arg-Arg-Met-Lys-Trp-Lys-Lys-Cys-NH₂ (11 μmol, 13 mg), in DMF (1.5 mL) was added Et₃N (1.5 μL). After stirring for 1.5 h the mixture was purified by preparative RP-HPLC (10 – 70 % MeCN gradient) to afford the pure title compound as a colourless solid (1.9 mg). Anal. RP-HPLC: t_R = 14.8 min (0 – 60 % MeCN gradient, purity > 96 %). DE MALDI-TOF MS: [M + H]⁺ = 2304.6 (C₁₀₇H₁₃₈N₂₄O₂₈S₃ = 2304.58).

Example 14

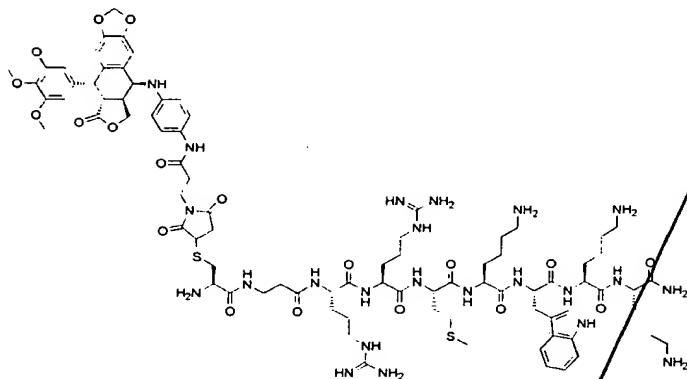
4'-(Succinimidopropionoyl)epipodophyllotoxin-(H-Cys-Arg-Arg-Met-Lys-Trp-Lys-Lys-Cys-NH₂)-2'-(succinimidopropionoyl)paclitaxel



To a solution of 4'-[succinimidopropionoyl-(H-Cys-Arg-Arg-Met-Lys-Trp-Lys-Lys-Cys-NH₂)]epipodo-phyllotoxin (2 μmol, 3.5 mg), 2'-(maleimidopropionoyl)paclitaxel (2 μmol, 2 mg) in DMF (1 mL) was added Et₃N (0.3 μL). After stirring for 1.5 h the reaction mixture was purified by preparative RP-HPLC (10 – 70 % MeCN gradient) to afford the pure title compound as a colourless solid (1.5 mg). Anal. RP-HPLC: t_R = 17.8 min (0 – 60 % MeCN gradient, purity > 98 %). DE MALDI-TOF MS: [M+H]⁺ = 2794.5 (C₁₃₄H₁₇₃N₂₃O₃₇S₃ = 2794.14).

Example 15

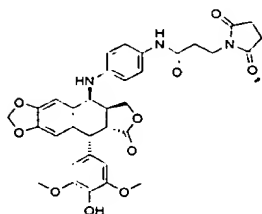
4'-Methoxy-4-[4''-aminoanilino-(succinimidopropionoyl)](H-Cys-βAla-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH₂)]epipodophyllotoxin



- 5 To a solution of 4'-methoxy-[4''-aminoanilino-(maleimidopropionoyl)] epipodophyllotoxin (7 μmol, 4.6 mg) and H-Cys-βAla-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH₂ (14 μmol, 16.3 mg) in DMF (1 mL) was added Et₃N (1 μL). After stirring for 1 h, the mixture was purified by preparative RP-HPLC (0 – 60 % MeCN gradient) to afford the pure title compound as a colourless solid (6.4 mg, 49 %). Anal. RP-HPLC:
- 10 $t_R = 15.2$ min (0 – 60 % MeCN gradient, purity > 98 %). DE MALDI-TOF MS: $[M + H]^+ = 1861.6$ ($C_{87}H_{125}N_{23}O_{19}S_2 = 1861.20$).

15 **Example 16**

4'-Demethyl-4-[4''-aminoanilino-(maleimidopropionoyl)]epipodophyllotoxin

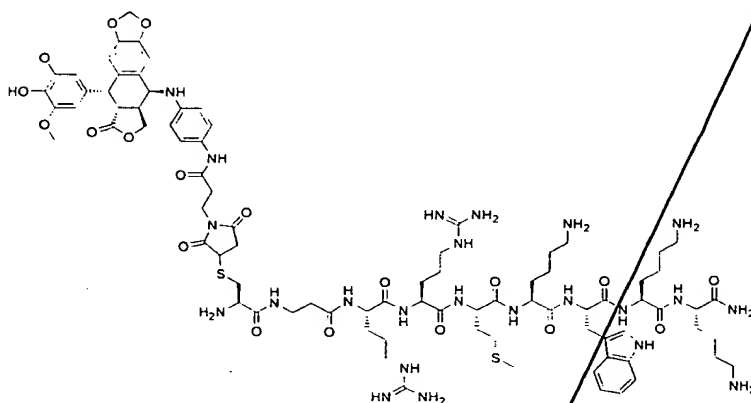


- To a solution of 4'-demethyl-4-(4''-aminoanilino)epipodophyllotoxin (24 μmol, 12 mg), 3-maleimidopropionic acid (49 μmol, 8.3 mg), and DIC (27 μmol, 3.4 mg) in
- 20

1:1 DMF/CH₂Cl₂ (2 mL) was added pyridine (10 µL). After stirring for 1 h, the reaction mixture was evaporated. The resulting light-yellow solid was purified by preparative RP-HPLC (10 – 70 % MeCN gradient) to afford the pure title compound as a colourless solid (5.3 mg, 34 %). Anal. RP-HPLC: t_R = 19.5 min (0 – 60 % MeCN gradient, purity > 96 %). ¹H-NMR (300 MHz, CDCl₃) δ : 2.65 (t, 2H, J = 7.3 Hz, CH₂), 2.98 (m, 1H, H₃), 3.17 (m, 1H, H₂), 3.79 (s, 6H, OCH₃), 3.93 (t, 2H, J = 7.0 Hz, CH₂), 3.99 (m, 1H, H₅, H₁₁), 4.38 (m, 1H, H₁₁), 4.58 (d, 1H, J = 4.95 Hz, H₁), 4.64 (d, 1H, J = 3.95 Hz, H₄), 5.96 (m, 2H, OCH₂O), 6.33 (s, 2H, H_{2'}6'), 6.49-6.53 (m, 3H, H₈, Ar), 6.74 (s, 2H, CH=CH), 6.75 (s, 1H, H₅), 7.33 (m, 2H, Ar).

10

4'-Demethyl-4-[4''-aminoanilino-(succinimidopropionyl)]-(H-Cys- β -Ala-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH₂)]epipodophyllotoxin



To a solution of 4'-demethyl-4-[4''-aminoanilino-(maleimidopropionyl)]-epipodophyllotoxin (8.3 µmol, 5.3 mg) and H-Cys- β -Ala-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH₂ (13 µmol, 15.6 mg) in DMF (1.5 mL) was added Et₃N (2 µL). After stirring for 1 h, the mixture was purified by preparative RP-HPLC (0 – 60 % MeCN gradient) to afford the pure title compound as a colourless solid (14.9 mg, 97 %). Anal. RP-HPLC: t_R = 13.7 min (0 – 60 % MeCN gradient, purity > 98 %). DE MALDI-TOF MS: $[M + H]^+ = 1847.1$ (C₈₆H₁₂₃N₂₃O₁₉S₂ = 1847.17).

20

Example 17

5 *Evaluation of etoposide and podophyllotoxin derivatives in topoisomerase II inhibition assay*

Topoisomerase II assay – Plasmid DNA (0.3 µg) was incubated at 37 °C with 4 units of purified recombinant human topoisomerase II in cleavage buffer (30 mM Tris.HCl, pH 7.6, 60 mM NaCl, 3 mM ATP, 15 mM mercaptoethanol, 8 mM MgCl₂) with or
10 without the addition of test compound (at 1mM, 100 µM, or 10 µM final concentration). Reactions were stopped by the immediate addition of SDS (1 % w/v final). Samples were treated with proteinase K (30 min at 37 °C) and extracted twice with an equal volume of 42:1 CHCl₃/*i*-amyl alcohol. After adding loading dye, samples were loaded to a 4 x TAE, 1 % agarose gel containing 0.5 mg/mL ethidium
15 bromide and electrophoresed for 16 – 24 h. Topoisomerase II inhibition was judged by the production of linear plasmid DNA, representing trapped cleavage intermediate, and by the ratio of substrate (supercoiled DNA) to product (relaxed DNA). A relaxation assay was performed identically, except that the reaction buffer was optimised for the detection of catalysis rather than cleavage, *i.e.* only 2 units of enzyme were used per
20 sample. The reaction buffer was 50 mM Tris.HCl, pH 8, 120 mM KCl, 0.5 mM ATP, 0.5 mM dithiothreitol, 10 mM MgCl₂. Topoisomerase II inhibition was judged by the ratio of substrate (supercoiled DNA) to product (relaxed DNA).

Table 8

Test Compound	Activity observed ^a
Etoposide	IC
Podophyllotoxin	-
4'-Demethylepipodophyllotoxin	IC
4'-Demethyl-4-(4''-aminoanilino)epipodophyllotoxin	I
H-βAla-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH ₂	-
4-[Succinimidopropionoyl-(H-Cys-βAla-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH ₂)]podophyllotoxin	-
4'-[Succinimidopropionoyl-(H-Cys-βAla-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH ₂)]epipodophyllotoxin	IC
4'-Demethyl-4-[acetyl-(H-Cys-βAla-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH ₂)]epipodophyllotoxin	IC
4'-Demethyl-4-[4''-aminoanilino-(succinimidopropionoyl)-(H-Cys-βAla-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH ₂)]epipodophyllotoxin	I

a) I denotes inhibition of relaxation of supercoiled plasmid by topoisomerase II. C denotes accumulation of topoisomerase II reaction intermediate.

5

Example 18

Comparison of full length and truncated penetratin as a vector for a drug moiety

- 10 In order to compare the cytotoxic biological effect on cancer cells (cell lines in Table 9) of the drug moieties applied using full length and truncated penetratin carrier moieties, appropriate podophyllotoxin-conjugates (podophyllotoxin-(16mer vector), 4-[succinimidopropionoyl-(H-Cys-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-Gly-Cys-Gly-NH₂)] podophyllotoxin; podophyllotoxin-7mer vector,
- 15 4-[Succinimidopropionoyl-(H-Cys-βAla-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH₂)]-podophyllotoxin), were exposed to cells at appropriate concentrations.

Serial dilutions of test compounds were applied to the cell lines. After incubation for 96 h, cytotoxicity was assessed using a standard sulforhodamine B (SRB) cell proliferation assay. IC₅₀ values are summarised in *Table 9*.

5 Table 9

	A2780	A2780 Cis ^R	CH1	CH1 Dox ^R	CH1 Taxol ^R	HCT116	HT29	KM12
podophyllotoxin, 16-mer ¹	0.55	0.48	0.46	0.49	0.21	0.52	0.54	0.48
podophyllotoxin, 7-mer ²	0.115	0.125	0.12	0.115	0.115	0.14	0.17	0.39

1. maximum tolerated dose by iv administration ion mice is ca. 40mg/kg (x mice)
2. maximum tolerated dose by iv administration ion mice is ca. 75mg/kg (x mice)

10 As can be seen from the Table, the truncated penetratin-podophyllotoxin conjugate is more effective in terms of anti-proliferative activity on tumour cells awhile exhibiting lower generalised toxicity.

15 Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

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